SUPPLEMENTATION OF LICORICE ROOT AND EPIMEDIUM IN A CONTROL OR HIGH FAT DIET AND THEIR EFFECTS ON A PRECLINICAL METASTATIC BREAST CANCER MODEL

BY

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DISSERTATION

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ABSTRACT

The natural decline in estrogen that occurs in women during menopause causes significant discomfort and decrease in quality of life for women. Botanical supplements contain multiple bioactive components, some of which have been identified to be phytoestrogenic and are used to remedy menopausal symptoms. However, due to their ability to mimic estrogen, the phytoestrogenic components contained in these botanical supplements may play a role in the progression of breast cancer. The majority of botanicals lack established data on safety and efficacy. They also present the likelihood for interactions to occur, such as with the high fat diet that is consumed in many Western countries.

Two such botanicals, licorice root and epimedium, which are used to alleviate menopausal symptoms, were studied for their impact on the progression of metastatic breast cancer in an animal model. In the licorice study, mice were supplemented with licorice root powder, extract or isoliquirtigenin for 2 weeks before and 3 weeks after cell injection. The effects of supplementation with the same licorice compounds alongside a high-fat diet were also explored. Mice fed licorice root powder and isoliquiritigenin were found to have a reduction in lung metastasis compared to control. In the high fat group, no changes in lung metastasis were found in animals supplemented with licorice root compounds compared with control. Although mild hepatocellular hypertrophy was observed in the liver of mice fed licorice root compounds, no significant adverse effects were found. In the epimedium study, mice were fed epimedium powder, extract or icariin for 12 weeks before and 4 weeks after cell injection. A second cohort also received the same epimedium treatments alongside a high fat diet. Supplementation with epimedium compounds had no effect on lung metastasis in the control groups, while only epimedium powder was found to decrease lung metastasis in the high fat cohorts. No adverse

effects due to epimedium supplementation were found. In this animal model, supplementation with licorice root or epimedium compounds did not have a promoting effect on breast cancer metastasis. Both licorice and epimedium possess properties that could be advantageous toward the development of novel pharmacological treatments. However, the safety of licorice root or epimedium supplement use remains uncertain due to the possibility of interaction with dietary components and should be approached with caution.

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CHAPTER 1: INTRODUCTION

Breast cancer (BC) encompasses the largest percentage of cancers in women worldwide and is the most significant contributor to cancer related mortality. In the U.S, breast cancer (BC) affects 12% of the female population and is the second leader of death in women. Though breast cancer mortality rates have gone down in the last ten years, there are still 40,000 American deaths due to breast cancer each year. Mortality incidence dramatically increases once a woman advances to late stage breast cancer, which involves metastasis to distant tissues such as lymph, lung and bone. Bone metastasis is a source of chronic pain for patients and has no standardized treatment. Micrometastasis, tiny undetectable tumors that are precursor to secondary tumors, were found in the bone at the time of diagnosis in approximately 30% of patients. The presence of bone micrometastasis were an indication for poor diagnosis and lower survival rate[1]. Modifiable lifestyle factors such as diet may factor into the development of detectable tumors in the bone from micrometastasis. Botanicals taken by the postmenopausal population pose a risk due to their phytoestrogen content. The high-fat diet that is consumed by American women has also been shown to increase the risk for breast disease.

Menopause, which is marked by a reduction in ovarian function and a decline in the production of estrogens, is a natural process of aging in women. During menopause, symptoms such as weight gain, hot flashes, night sweats, mood changes, and diminished sexual function[2] may appear as a result. In order to alleviate these symptoms, Hormone Replacement Therapy (HRT), synthetic estrogens in the form of a pill, skin patch, or topical application was commonly prescribed. Although HRT is effective for menopausal symptoms, use has declined since the emergence of evidence linking HRT with an increased risk of breast cancer, as well as other possible health risks[3,4]. Consequently, postmenopausal women have sought alternatives in order

to relieve their symptoms. Botanical supplements are a popular form of alternative therapy thought to improve menopausal symptoms through their phytoestrogenic properties[5]. A study at the University of Illinois at Chicago clinics reported that 79% of peri- and postmenopausal women were using botanical dietary supplements[6]. Botanicals are perceived to be safe for consumption because they provide a natural source of phytoestrogens. However, the safety and efficacy of ingesting botanical phytoestrogens is undetermined.

The phytoestrogen genistein contained in soy seems to play a protective effect when consumed as a regular part of the diet[7], but may exacerbate the growth of breast tumors when consumed as a supplement in later life[8]. The phytoestrogens found in botanicals structurally resemble estrogen and therefore have the capacity to bind to estrogen receptors (ER). Estrogens act as ligands to bind to estrogen receptor (ER) to modulate gene responses. While estrogens play a crucial role in the female body by regulating menstruation, sexual development, sexual reproduction, and metabolism[9], binding to either of the estrogen receptors, ERα or ERβ, can contribute to the progression of breast cancer. ERα is thought to modulate most of the pro-oncogenic action while ERβ plays a moderating role[9,10]. Botanicals have a higher affinity toward ERβ than ERα, but since the ERs share a large homology, possess the potential to turn on or off genes that regulate cancer growth[11].

The main types of estrogen in the body are 17β-estradiol (E2) as well as estrone (E1) and estriol (E3). In premenopausual women, E1 and E2 are secreted by the ovaries throughout the menstrual cycle, with some coming from the adipose tissue or adrenal glands, while E3 is secreted by the placenta during pregnancy[9,10]. In postmenopausal women, estrogen synthesis in the ovaries is low, and the primary source of estrogen is synthesized by aromatase, which lies in the adipose tissue, as well as in ovaries, placenta, bone, skin and brain[12,13]. Therefore, for

postmenopausal women who are obese, estrogen synthesis in the adipose becomes more significant. Obesity is a growing problem in the U.S and is an important contributor to survival outcomes in breast cancer patients. Patients who were classified as overweight or obese prior to diagnosis had a higher risk of mortality due to breast cancer[14]. When compared with normal individuals, postmenopausal women who were overweight or obese and had been diagnosed and treated for breast cancer were shown to have a greater incidence of recurrence or disease progression[15]. High fat diets that are consumed by the American population are often associated with the development of obesity.

Studies regarding the role of high dietary fat consumption and breast cancer are inconsistent, but some suggest a positive correlation. Higher incidence of invasive breast cancer was found in women with higher intakes of total fat and saturated fat when compared with women with the lowest intakes of fat[16,17]. Women consuming greater amounts of saturated fat, monounsaturated fat, and total fat before breast cancer diagnosis were also reported to have a higher risk of death[18]. A diet high in dietary fat, along with the use of botanical supplements for menopausal symptoms, may have unintended consequences for those with or at high risk for developing breast cancer. The progression of breast cancer to metastatic breast cancer in the bone can be life-debilitating and accounts for a large portion of deaths. Botanicals exhibit estrogenic properties that may protect women from breast cancer metastasis or may stimulate the growth of developing tumors. The interactions exerted by a diet high in fat can complicate the situation. The effects of consuming botanical supplements and a high fat diet in combination or alone could instigate the growth of breast tumors and metastasis, and there is a need to delineate those outcomes.

My research project focuses on two botanicals, licorice root and epimedium, which are available on the retail market and consumed by women for menopausal symptoms. Although a number of studies have shown the effects of these botanicals on breast cancer, there is limited knowledge on their effects on breast cancer metastasis. Many animal studies have shown a relationship between high-fat diets and increased breast cancer incidence. These studies are conducted using a standard rodent "chow" diet, which is a relatively healthy diet with a low fat content. The dietary fat content in these studies do not account for the higher amounts of fat (approximately 33% of total kcal according to the CDC) that an average American woman would be consuming. The combination of a high-fat diet with botanical supplementation has not been investigated in animals or in humans. Furthermore, no studies have taken into account the possibility of an interaction between botanical supplementation and the level of fat consumed in a breast cancer animal model. In order to make the study as relevant to our target population as possible, we give animals levels of botanical and high levels of fat that are achievable in humans. Using an animal mouse model will help us characterize possible clinical outcomes in a breast cancer patient. Using a cell line that spontaneously and preferentially metastasizes to the lung when injected into the bone of the animal allows us to measure the degree of metastasis by counting the number of metastatic lung nodules. As of such, no studies have utilized an in vivo mouse bone model to mimic the metastasis from bone to lung to investigate the effects of botanicals. In addition, the potency of whole botanical (in dried powder form), botanical extract, and bioactive component in a breast cancer metastasis model will be compared.

The long-term goal of this project was to assess the potential health risk and/or benefit of botanical consumption in women who are at risk for breast cancer by examining the effect of botanical supplementation of licorice root and epimedium on BC growth and metastasis. The

objective of these studies were to determine how botanical consumption of licorice and epimedium affects breast cancer metastasis in a preclinical breast cancer metastasis model representative of a woman who is at risk for breast cancer.

The hypothesis for these studies are that licorice root and epimedium compounds would reduce the metastasis of 4T1 murine breast cancer from bone to lung in a preclinical OVX Balb/C model and that a high fat diet (HF) compared to control diet (C) would negate or alter those effects.

The specific aims for this project are as follows: 1) To investigate the effects of licorice and epimedium, in the form of whole botanical (in dried powder form), botanical extract, and bioactive component on breast cancer metastasis from bone to lung in a murine model, 2) To determine if there is an interaction effect between licorice or epimedium with dietary fat

This thesis is divided into five chapters. Chapter 2 goes into detail about the general and estrogenic properties of licorice root and epimedium, as well as their relationship to breast cancer. More information will also be provided on the high fat breast cancer models used in other animal studies. Chapter 3 will describe the findings from the licorice root study while Chapter 4 will narrate results and discussion from the epimedium study. Chapter 5 will further summarize the research findings and their implications for future work.

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CHAPTER 2: LITERATURE REVIEW

Estrogens and Cancer

Estrogen is a crucial hormone that is responsible for development, maintenance and regulation in the female body. The main types of estrogen in the body are 17β-estradiol (E2) as well as estrone (E1) and estriol (E3). In premenopausual women, E1 and E2 are secreted by the ovaries throughout the menstrual cycle, with some coming from the adipose tissue or adrenal glands, while E3 is secreted by the placenta during pregnancy[1,2]. In postmenopausal women, estrogen synthesis in the ovaries is low, and the primary source of estrogen is synthesized by aromatase, which lies in the adipose tissue, as well as in ovaries, placenta, bone, skin and brain[3,4]. For postmenopausal women who are obese, estrogen synthesis in the adipose may be more significant. Excess exposure to estrogens is also associated with greater risk of breast cancer[5]. Multiple epidemiological studies[6,7] as well as randomized controlled trials[8,9] have supported the original findings from the Women's Health Initiative Study that hormone replacement therapy (HRT) in the forms of combined estrogen/progesterone therapy as well as estrogen therapy alone were associated with a higher incidence of invasive breast cancer in current users and those who had stopped use 1-4 years before diagnosis[10]. It is postulated that mechanisms of estrogen on breast carcinogenesis include the metabolism of estrogen into genotoxic or mutagenic metabolites and the stimulation of tissue growth through estrogen signaling[11]. Estrogen acts by binding to estrogen receptor (ER), which is found in many tissues of the body, like uterus, ovary, mammary gland, prostate, lung and brain. Activated estrogen-ER complex causes changes in protein structure and allows interaction with coactivators or corepressors. The complex can regulate gene transcription by directly binding to estrogen response elements (ERE) located in promoters of target genes or through protein-protein

interactions with other transcription factors. Alternatively, phosphorylation of ERs by activated kinases stimulated by growth factor receptors can also lead to activation. The activated ERs are also subject to post-translational modifications that can affect their activity[1,12,13]. To add to the complexity of estrogen signaling, the two types of estrogen receptor, ERα and ERβ, share a large homology (~59%) and perform opposing roles in cellular processes. In general, interactions with ERα are linked to proliferative effects while interactions with ERβ are linked to inhibition of growth. ERα expression is often increased in early breast tumors while ERβ is reduced[14]. The spacious ligand cavity of ER allows for binding of molecules with some resemblance to estrogen[15].

Figure 2.1. Estrogen Receptor Ligand Activation Pathways. Adapted from Heldring *et al*[13].

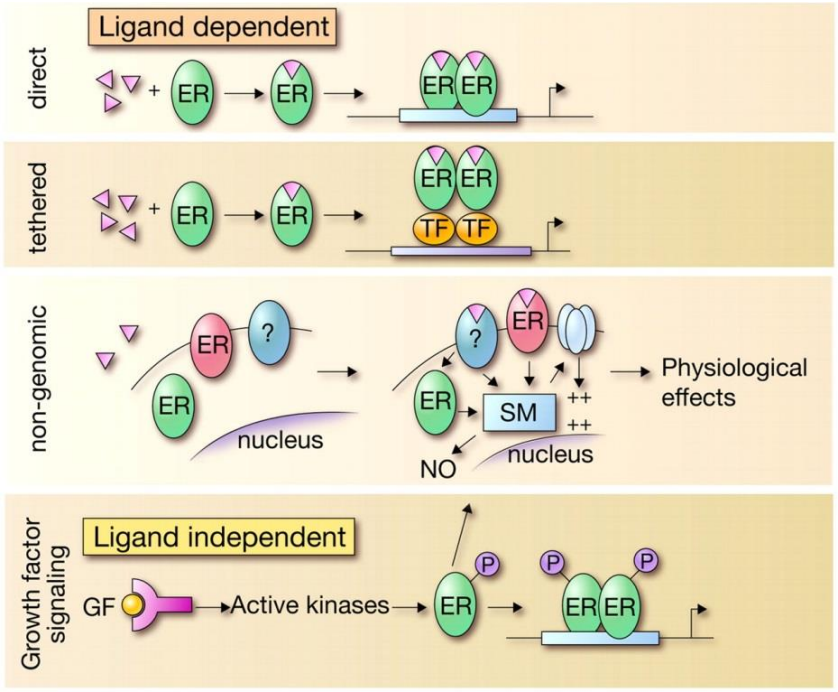
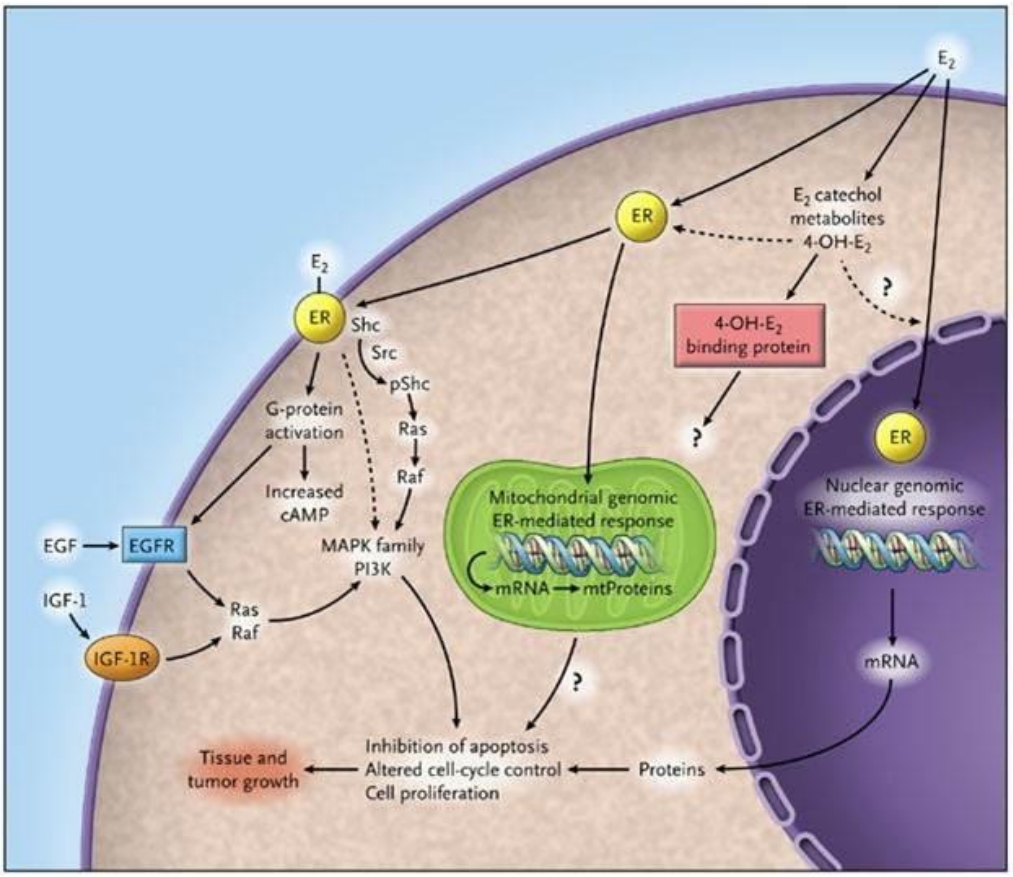


Figure 2.2. Estrogen Receptor Signaling Pathways. Abbreviations: cAMP denotes cyclic AMP, E_2 estradiol, 4-OH-E_2 4-hydroxyestradiol, ER estrogen receptor, EGF epidermal growth factor, EGFR epidermal growth factor receptor, IGF-1 insulin-like growth factor 1, IGF-1R insulin-like growth factor 1 receptor, MAPK mitogen-activated protein kinase, mRNA messenger RNA, MPI3K phosphoinositide 3 kinase, mtProteins mitochondrial proteins, and pShc phosphorylated Shc protein. Dashed-line arrows indicate putative pathway. Adopted from Yager and Davidson[11].

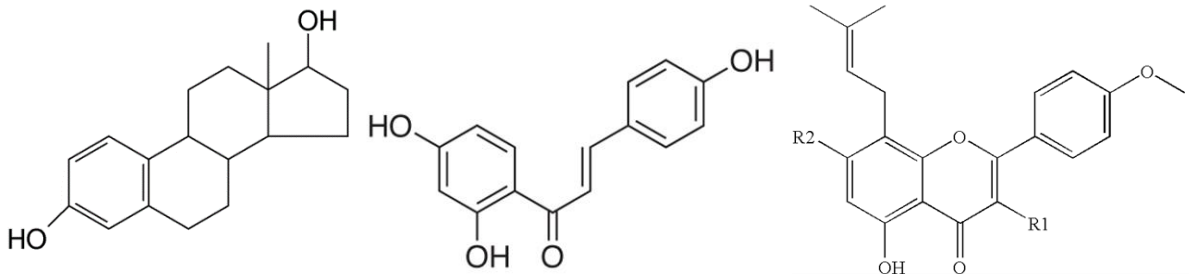


Botanical Phytoestrogens

As such, HRT is used to relieve symptoms such as hot flashes, night sweats, insomnia and others by menopausal women and women who have undergone adjuvant therapies for breast cancer. With the mounting evidence that the risks of using of HRT outweigh its benefits, women have sought alternative forms of relief through natural botanical supplements, which contain phytoestrogens. Phytoestrogens have similar structures to estrogen and tend to have low

affinities to ER when compared with estrogen[16]. Botanicals also have a higher affinity toward ERβ than ERα [17]. Studies investigating the potential benefits of phytoestrogens against the development of hormone-responsive cancers have yielded ambiguous results. Epidemiological studies have found that the rates of breast cancer in Asian countries are much lower compared to the Western hemisphere[18,19], presumably due to higher intakes of soy and isoflavones[20-22]. Other studies have found no relationship between the consumption of phytoestrogens such as soy and the risk of breast cancer[23,24]. However, breast cancer patients and survivors are not recommended to use botanical phytoestrogens. Due to the vast array of available phytoestrogens and the diversity of their actions, more research is needed to determine their safety, especially towards breast cancer. Two botanicals of interest, licorice root and epimedium, will be discussed further in the following portions of this review.

Figure 2.3. Chemical structure of isoliquiritigenin, a bioactive estrogenic component of licorice root and chemical structure of icariin, a bioactive estrogenic component of epimedium, in the orientation that is most like estradiol.



Estradiol **Isoliquiritigenin** **Icariin**

Licorice Root Historical and Commercial Use

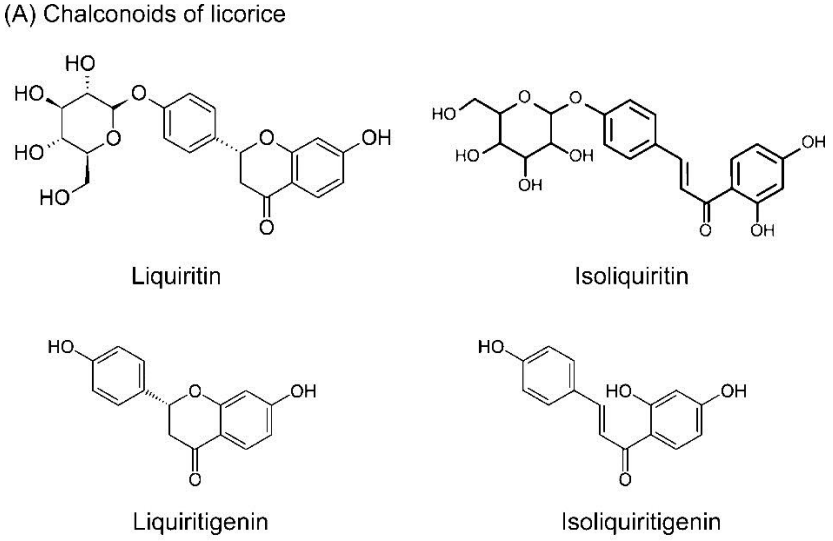
Licorice root (genus name Glycyrrhiza) has a long history of use in Western and Eastern cultures. Licorice (LR) has been used as a remedy for ailments such as stomach ulcers, bronchitis, sore throat and hepatitis, as well as a sweetening and flavoring agent. Today, it is

mainly grown in Greece, Turkey and Asia, is still used in folk medicine and Chinese medicine[25], and is a popular ingredient for tobacco and confectionary products. Commercial preparation of licorice products involves the drying of the root, the mashing and boiling of the root into pulp, and removal of solids to form a paste, which can then be vaccuum dried into blocks or dried to powder, depending on the application[26]. The primary flavor compound found in licorice root is glycyrrhizin, which is also an active compound. Large amounts and long-term use have been known to cause edema, hypertension and hypokalemia[27]. Licorice root and its constituents have been reported to be antioxidative, anti-inflammatory, antiviral/antimicrobial, and anticarcinogenic. Licorice root and its derivatives are considered GRAS by the U.S Food and Drug Administration. The three main species of licorice grown in China are Glycyrrhiza glabra, Glycyrrhiza uralensis, and Glycyrrhiza inflata. Licorice root contains multiple bioactive compounds comprising saponins, flavonoids, isoflavones, coumarins, and stilbenoids[28]. Of these, the triterpenoid saponin glycyrrhizin (consisting of potassium and calcium salts of glycyrrhizic or glycyrrhizinic acid, and glycyrrhetinic acid) is considered to be present in highest amounts and contributes to the sweetness of licorice root. Other widely researched components include the flavonoids liquiritigenin, isoliquiritigenin, and licochalcones, as well as the isoflavonoid derivatives glabridin and glabrene, all of which have been reported to have estrogenic activity[29-31]. Glycyrrhizin has not been found to be teratogenic, genotoxic, or toxic to fetal development[32]. Glycyrrhizin was evaluated in 2005 at the Joint FAO/WHO Expert Committee on Food Additives and it was determined that a consumption of 100 mg/day would not cause adverse effects in most adults[26]. Licorice root and its constituents have been reported to be antioxidative, anti-inflammatory, antiviral/antimicrobial, and anti-carcinogenic. Overall, licorice root is widely used and is relatively safe for consumption.

Isoliquiritigenin Bioavailability and Metabolism

Isoliquiritigenin (ILQ) is a compound of interest that is found in licorice root and is a precursor chalcone which forms liquiritigenin (LQ) after cyclization. Liquiritin and isoliquiritin are glycones of ILQ and LQ. In mice, isoliquiritigenin was detectable in plasma five minutes after oral administration, suggesting that it was quickly absorbed through the gastrointestinal tract. Tissue/plasma ratios of ILQ after were greater than unity (T/P >1) in liver, spleen, stomach, small intestine, large intestine, kidney, and lung, indicating high distribution per oral administration. Mean plasma concentrations of ILQ and LQ after iv doses of 2.5-20 mg/kg ILQ and oral doses of 10-100 mg/kg ILQ were not significantly different[33]. Plasma concentration-time profiles of ILQ after venous and oral administration was similar in rats; however, LQ was below the detection limit 30 min after adiminstration. It was estimated that oral absorption of ILQ was about 92% absolute bioavailability was about 11.8%[34]. In human liver microsomes, isoliquiritigenin was mainly converted to liquiritigenin upon Phase 1 metabolism, as well as six other metabolites (2′,4,4′,5′-tetrahydroxychalcone, sulfuretin, butein, davidigenin, *trans*-6,4′-dihydroxyaurone, and *cis*-6,4′-dihydroxyaurone)[35]. Cytochrome P450 CYP 2C19 and UDP-glucoronysyltransferase 1A were primarily responsible for the second phase of metabolism for ILQ. Phase 2 metabolism in human hepatocytes produced LQ and five monoglucoronides of ILQ, with UGT1A1 and UGT1A9 producing the most common metabolite. Some metabolites were formed by human intestine and kidney microsomes as well[36].

Fig 2.4. Chemical Structures of Licorice Chalconoids. Adapted from Kao *et al*[37].



Licorice Root and Estrogenicity

Estrogenic activity of licorice root has been established in many studies. Licorice root can be fractionated to licorice root extracts using various solvents: DMSO for compounds of differing polarities, hexane for non-polar phytochemicals, ethyl acetate and methanol for compounds with intermediate polarity, and hot water for polar phytochemicals. Contrasting effects on cell growth were seen using extracts made from different solvents. DMSO and ethyl acetate extracts showed biphasic effects on MCF-7 growth, where low concentrations promoted ER-dependent growth and high concentrations inhibited growth independent of ER. Hexane extracts had no effect on growth, while methanol and water extracts promoted ER-independent growth[38]. Fractionated ethyl acetate extracts of licorice root all exhibited estrogenic responses that were ER-mediated due to an ablation of activity in the presence of an ER-antagonist and a lack of activity in cells that did not contain ER. Glabrene-rich fractions preferentially bound to ERalpha to activate, whereas glabridin behaved as an ERα antagonist[39]. It has been suggested that the large range of compounds found in licorice root may exert both estrogenic and ant-

estrogenic effects. The main phenolic compounds found in licorice root are liquiritin and its chalcone derivative isoliquiritin, as well as liquiritigenin and its chalcone derivative isoliquiritigenin[40]. ILQ can bind to ERα and ERβ of various cell types and displayed a biphasic effect on proliferation of MCF-7 cells[41]. Liquiritigenin, the metabolite of isoliquiritigenin, has very low relative binding affinity to either of the ER's when compared with genistein, but preferentially binds and activates ERβ[42]. Although liquiritigenin has only a 20-fold greater binding affinity to ERβ over ERα, it selectively recruits coactivators to ERβ, making it a more potent and effective ligand of ERβ[43]. In addition to the agonistic actions of isoliquiritigenin and liquiritgenin on ER, four other compounds isolated from licorice root (calycosin, methoxychalcone, vestitol, and glycycoumarin) were identified as ER agonists and three other compounds (glabridin, glyasperin C, and glicocoricone) were identified as ER antagonists.[44] These findings suggest that collectively, components of licorice root can act as selective estrogen receptor modulators (SERM)s, which may explain the differences in activity reported from previous studies.

Licorice Root and Cancer

Very few studies have looked at the pharmacological properties of licorice root powder alone. This may be attributed to the potential toxicity of glycyrrhizin present in whole licorice root, while in Chinese medicine, licorice root is most often used in combination with other herbs. Most studies have investigated the activities of licorice root extract or isolated compounds from licorice root. Licorice root extracts were shown to inhibit proliferation of colon cancer cells[45], decrease the metastatic potential of prostate cancer cells[46], and induced apoptosis of breast cancer cells[47]. However, it should be noted that the triterpene saponin content of licorice roots

differ depending on the species used[48] and cytotoxicity of licorice roots can vary based on geographical area and timing of harvest[49].

Isoliquiritigenin is present in all Glycyrrhiza species and has been widely studied for cancer prevention. ILQ was able to suppress tumor growth of colon, prostate, lung, and breast cancer cells. ILQ was proposed to decrease cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS), two factors that are considered relevant to colon cancer. Protein levels of COX-2 and iNOS were decreased in macrophages with ILQ treatment. In rat colons, ILQ inhibited formation of total and large carcinogen-induced pre-neoplastic abberant crypt foci (ACF)[50]. In adenoid cystic carcenoma cells, isoliquirtigenin caused apoptosis. Membrane-mediated apoptosis is regulated by proteins of the Bcl-2 family. Bcl-2 works to protect the mitochondrial outer membrane while Bax promotes the release of pro-apoptotic facters such as cytochrome c and Smac/Diablo. Isoliquiritigenin was found to induce apoptosis of both rat and human prostate cancer cells, which was correlated with increased levels of Bax protein, as well as cytochrome c and Smac/Diablo. ILQ was not toxic to intestinal epithelial cells[51]. Tumors are thought to produce arachidonic acids, which are metabolized by COX, lipoxygenase, and cytochrome P50 (CYP) to eicosanoids such as prostaglandin E_2 (PGE_2), leuokotriene B_4 (LTB_4). Eicosanoids can then promote cancer cell survival by activating phosphatidylinositol 3-kinase (PI3K)/Akt pathway. It was established that ILQ decreased cell viability and induced apoptosis in breast cancer cells MCF-7 and MDA-MB231 by significantly lowering mRNA levels of sPLA2, COX-2 and CYP4A1. ILQ also suppressed secretion of PGE_2 and lowered Akt kinase activity, indicating downregulation of AA metabolic network and deactivation of PI3K/Akt pathway. Additionally, tumor growth MDA-MB-231 xenografts in nude mice were inhibited with treatment of isoliquiritigenin at 50 and 100 mg/kg IP dosages[52].

Bone resorption by osteoclasts releases growth factors from bone matrix that may contribute to proliferation of cancer cells in the bone. Roasted licorice root extracts inhibited RANKL secretion but had no effect on OPG. The ethanolic extract of roasted licorice root reduced the viability of MDA-M231 cells in a dose-dependent manner. Treatment with licorice root extract by gavage (0.5, 1, or 2 mg/kg body weight) reduced tumor growth and extent of bone destruction in tibia of animals injected with MDA-MB231 cells.[53]

Licorice Root and Metastasis

The anti-metastatic effects of ILQ in prostate cancer cells were investigated. ILQ inhibited basal and EGF-induced migration and invasion of prostate cancer cells, as well as adhesion. ILQ also reduced MMP-2 and VEGF in prostate cancer cells. Binding of EGF to EGF receptor leads to downstream activation of several signaling molecules. ILQ was found to inhibit phosphorylation of Akt, JNK, and c-Jun , but not p38 MAPK or ERK1/2[54]. In lungs of mice injected with renal carcinoma cells, ILQ treatment significantly reduced the number of metastatic nodules at 2 and 10 mg/day for 10 days.[55] Licochalcone E, another phenolic constituent of licorice root, reduced tumor growth with oral administration of 14 mg/kg and tumor nodules in lung with oral administration of 7-14 mg/kg in mice injected with 4T1 cells[56]. Proposed mechanisms are decreased angiogenesis through inhibition of VEGF expression due to accelerated degradation of upstream HIF-1α proteasome in MDA-MB-231 cells[57]. Further support of ILQ's anti-metastatic capabilities was demonstrated with a decrease in surface lung tumors with ILQ treatment of 10 and 20 mg/kg BW in mice injected with MDA-MB-231 cells. These results were correlated with a decrease in levels of PGE2 and 20-HETE, phospho-Akt, MMP-2 and MMP-9 in primary tumors of animals treated with ILQ[58].

Epimedium Historical and Commercial Use

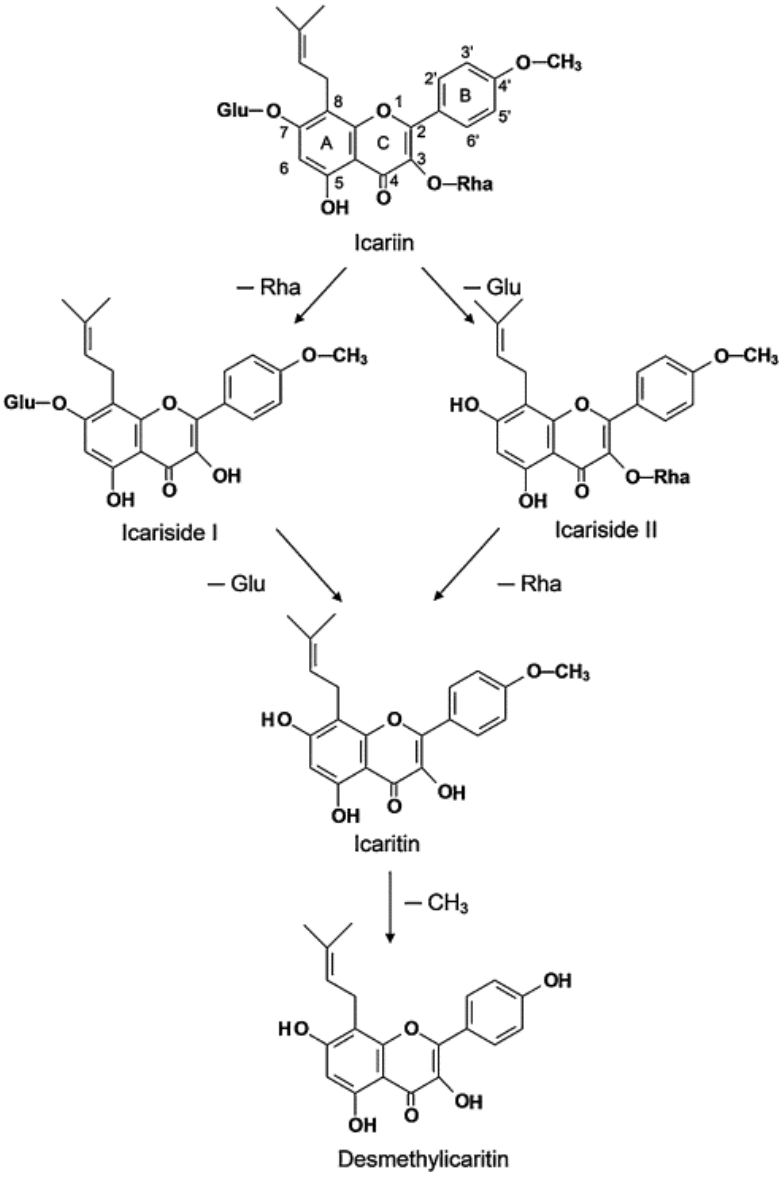
Epimedium, or Herba Epimedii, is a genus of 21 species of plants that are grown in Asia and the Mediterranean. It is also known commonly as horny goat weed, barrenwort, and yin yang huo. The leaves and stems of Epimedium are used as a supplement, historically as a remedy for kidney dysfunction, impotence, and as an aphrodisiac or stimulant. Species used for medicinal purposes are *Epimedium sagittatum, Epimedium brevicornum, Epimedium wushanense, Epimedium koreanum*, and *Epimedium pubescens.* Epimedium has been investigated for its abilities to treat sexual dysfunction, osteoporosis, cardiovascular disease, asthma, bronchitis, and sinusitis[59]. Long term use of Epimedium in Chinese medicine has been safe when consumed orally at around 5 g or at around 300 ml of extract daily for up to 6 months. Individuals with pre-existing conditions such as cardiovascular disease, hypertension, muscoskeletal disorders, bipolar disorder, hypothyroid conditions, and respiratory disorders are recommended not to take Epimedium[60]. Excessive intake of epimedium can cause vomiting, dizziness, thirst and nosebleed[61].

Bioavailability and Metabolism

Epimedium consists of many bioactive compounds and is a rich source of prenylflavonoids. Icariin, the principal component of Epimedium, contains a glucose and a rhamnose residue. Partial deglycosylation results in icariside I and icariside II, and further deglycosylation results in icaritin. Other isolated and studied compounds with estrogenic properties include ikarisoside A[62], baohuoside I and baohuoside II[63], breviflavone B[64], epimedin B and epimedin C[65]. A mixture of icariin, icariside I, icariside II, and icaritin were contained in an ethanolic extract of Epimedium. All derivatives appeared in the nanomolar range after administration of 100, 300 and 600 mg/kg/BW of epimedium extract to rats. Although icariin held the highest flavonoid content in epimedium, it was quickly metabolized to icariside II by

first-pass deglycosylation. After digestion with glucoronidase, only icariside I, desmethylicaritin, and icaritin were detectable. Additionally, saturation of icaritin was observed at higher levels of epimedium extract administration[66].

Figure 2.5. Structures of icariin and its derivatives. Adapted from Wong *et al* [66].



Epimedium and Bone Health

Osteoporosis occurs when the rate of bone resorption exceeds the rate of bone formation. The main players in bone maintenance are osteoblasts and osteoclasts. Osteoblasts produce receptor activator of NFκB ligand (RANKL), whose receptor (receptor of NFκB) is found on osteoclasts. Osteoblasts also secrete osteoprotogerin (OPG), a decoy peptide which prevents RANKL from binding to RANK. Upon binding of RANKL, activation of nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) signaling pathways increases transcription factors necessary for differentiation of osteoclasts. Osteoclast activation and differentiation leads to the resorption of bone. Estrogen signaling plays a large role in bone metabolism by regulating RANKL/OPG ratio, inhibiting osteoblast apoptosis, and increasing osteoclast apoptosis[67].

Epimedium has been reported to have beneficial effects on osteoporotic bone. Icariin is able to act similarly to estradiol and increase the expression of OPG/RANKL ratio as well as stimulate cell proliferation in UMR-106 cells. However, unlike estradiol, icariin did not induce ERE-dependent luciferase activity in UMR-106 cells via ERs, but did increase ERα phosphorylation at Ser118, a major site for ligand-independent activation of ER [68]. Treatment of icariin in ovariectomized-induced osteoporotic mice resulted in upregulation of OPG and downregulation of RANKL gene expression[69]. Four components extracted from Epimedium, icariin, baohuoside-1, epimedin B and sagittatoside A had stimulatory effecst on cell proliferation in osteoblast-like UMR-106 cells. These effects were abolished by co-treatment of cells with ICI 182,780. In a binding assay for ERα and ERβ, none of these compounds changed the binding of E2 to ERs. It was concluded that the stimulatory effect was likely due to interaction with ER, but not through its binding. ERα protein expression was suppressed in

UMR-106 cells with treatment of E2, baohuoside- I and epimedin B, and induced with treatment of icariin[70].

Epimedium and Estrogenicity

It has already been established that Epimedium exhibits estrogenic activity by regulating bone cells. Epimedium and compounds isolated from epimedium have also been reported to have varying levels of estrogenicity in other cell types. Icaritin and desmethylicaritin, but not icariin, strongly stimulated proliferation of MCf-7/BUS cells. Increase in cells in S phase and significant increase in G2/M population similar to effects of estradiol. Treatment with icaritin or desmethylicaritin increased estrogen receptor-regulated progesterone receptor and PS2 mRNA levels. These effects were reversed by ICI 182, 780[71]. Icaritin and desmethylicaritin increased MCF-7 cell proliferation. Cell proliferation was antagonized by ICI 182, 780. Progestogen Receptor mRNA levels increased after treatment with icaritin and desmethylicaritin. These effects were not seen with icariin[72]. Icaritin inhibited the growth of breast cancer MCF-7 cell sand MDA-MB-453 cells[73].

Biosynthesis of estrogen from androgens in humans is catalyzed by aromatase CYP19A1. In humans, the major source of estrogen in premenopausal women comes from human ovarian granulosa cells. Treatment of KGN cells with a crude extract of Epimedium brevicornum was found to promote 17β-estradiol biosynthesis. These effects were also seen with treatment of KGN cells with icariin. Icariin treatment increased aromatase protein expression. The increase in 17β-estradiol biosynthesis was abolished with aromatase inhibitor letrozole[74].

Epimedium and Breast Cancer

Mice treated with icariin and icaritin via IP injection at 100 mg/kg three times a day for seven days after tumor inoculation treated had reduced tumor volume and contained splenocytes

with a significantly higher percent of IFN-y producing $CD8^+$ T cells compared with control mice[75]. Athymic OVX female nude mice with MCF-7 breast cancer xenografts were fed high dose of epimedium extract (5000 mg/kg) for 16 weeks. Animals fed epimedium showed regression of tumors that were smaller compared to control animals after 16 weeks.ERα protein was reduced in tumor cells of animals fed 5000 mg/kg epimedium extract[76]. Icaritin at low concentrations stimulated growth of MCF-7 cells while higher concentrations suppressed MCF-7 proliferation. Icaritin with estradiol suppressed estradiol-stimulated cell growth. The combination of estradiol and icaritin significantly reduced GREB1 mRNA, a critical hormone-dependent regulator of breast cancer growth. Icaritin was found to competitively bind to AhR and acted as an AhR agonist. Icaritin reduced ERα protein levels in tumors of athymic OVX nude mice. Treatment with icaritin did not increase growth of MCF-7 breast tumor xenografts in athymic nude mice[77].

Icariin induces apoptosis through NFκB pathway

Icariin has been shown to suppress NFκB through a non-classical pathway, leading to downstream decrease in anti-apoptotic factors. Icariin treatment induced apoptosis in both colorectal cancer cell line HCT116 and gallbladder cancer cell GB-SD. Icariin induced apoptosis in a dose dependent manner in human gallbladder carcinoma cell line via caspase-3 activity and decrease in antiapoptotic molecules Bcl-2, Bcl-xl and survivin through inhibition of NFκB. It also enhanced caspase-3 activity, induced G_0-G_1 phase arrest and suppressed the expression of Bcl-2, Bcl-xL and surviving proteins. Icariin inhibited constitutive NFκB as well as gemcitabine-induced NFκB activity. Icariin also potentiated the effects of gemcitabine in vitro and in vivo[78]. Icariin inhibited cell proliferation in a dose dependent manner in colorectal cancer cells HCT116 and HT29, although HCT116 cells were more sensitive than HT29. This effect was potentiated

when cells were treated with both icariin and radiation. When cells were pre-treated with icariin before exposure to radiation, NFκB was suppressed in comparison with cells that were exposed to radiation alone[79]. Cell viability of human non-small cell lung cancer A549 cells was inhibited by treatment of baohuoside I (isolated from Epimedium Koreanum), which resulted in apoptosis after 24 hours treatment. It was found that treatment with baohuoside I induced ROS production, which was diminished with ROS scavenger NAC incubation, reducing apoptosis levels[80].

High Fat Animal Model

Epidemiological studies investigating the relationship between dietary fat and breast cancer are inconclusive. In rodent models, the relationship seems to be clearer, with a number of studies showing a link between high-fat diets and cancer growth. High fat diets were found to increase tumor growth in rodents when using syngeneic 4T1 mammary cancer cells, human MCF-7 mammary cancer cells, CT26 and HT-29 colon cancer cells. The Park group used the mammary mouse cell line 4T1 in the BALB/c mouse model fed a diet containing 60% kilocalories from total fat fed over 16 weeks. They showed that a 16-week feeding was sufficient to induce low-grade inflammation in this model, as noted by the increase in macrophage infiltration of the gonadal fat pad as well as an increase in serum cytokines (C5a, sICAM-1, IL-16, M-CSF, TIMP-1, and TREM-1) in the mice fed high-fat diet[81]. There were only slight increases in body weight and gonadal adipose deposits between mice fed high-fat compared with mice fed control diet, while other adipose deposits were undetectable. Mice fed a high-fat diet implanted with 4T1 mammary tumors had reduced survival rate, increased solid tumor growth, and increased lung and liver metastasis compared with those fed a control diet. In athymic mice implanted with MCF-7 tumors, consumption of high-fat diet led to decreased numbers of splenic NK cells and altered regulation of cell cycle[82]. Similar results were observed using colon cancer

cell lines. In mice injected subcutaneously with CT26 colon cancer cells, mice fed a high-fat diet had significantly increased tumor growth and progression[83]. High-fat diet stimulated leuokocyte infiltration and angiogenesis in tumor tissues, seen by significant increases in serum VEGF and increased hemoglobin content in tumor tissues, Animals fed high-fat diet also had increased serum levels of growth factors and pro-inflammatory cytokines/chemokines. Mice injected with CT26 cells had significantly greater numbers of lung tumor nodules. In BALB/c nude mice with HT-29 cell xenograft, tumor tissues of mice fed high-fat diet had higher expression of PCNA and COX-2, which were confirmed with suppression of nuclear p21 levels and increased phosphorylation of ERK1 and mTOR proteins, suggesting activation of oncogenic pathways phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR and mitogen-activated protein kinase (MAPK)/ERK signaling cascades with the consumption of a high fat diet.

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CHAPTER 3: LICORICE STUDY

Abstract

Licorice root, used for many centuries to treat various ailments, especially in Asian cultures, is a botanical supplement used by women to improve menopausal symptoms. Although the perception is that botanical supplement usage is safe, they contain phytoestrogens that are weakly estrogenic and are able to bind to estrogen receptors (ERs). This is especially dangerous in women who are at risk for developing breast cancer or have existing microtumors which have been undiagnosed. This study aimed at determining the consequences of licorice root compound supplementation on breast cancer metastasis in a preclinical breast cancer model representative of late-stage breast cancer in women. We fed licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a control (C) or high fat (HF) diet to ovariectomized Balb/c mice injected with 4T1 murine cells in the bone and measured the degree of metastasis to the lung. We demonstrated that licorice root consumption has the potential to modulate the degree of metastasis from bone to lung in an animal model fed a C diet. These effects were negated in a HF diet. In addition, licorice root compounds had no proliferative effects on uterine growth or terminal end bud enlargement in mammary gland. Although growth promoting effects on metastatic breast cancer were not observed with supplementation of licorice root and its constituents, more research to ascertain how licorice root compounds interact with other dietary components and in different tissues would help direct appropriate consumption of licorice root and its constituents in women with breast cancer.

Introduction

Breast cancer (BC) affects 12% of the female population and is the second leader of death in U.S women, with an estimated 60,290 new cases diagnosed in 2015[1]. However, survival

rates have improved drastically, with more than 2.8 million BC survivors living in the U.S. Complications from BC usually arise as a result of metastasis, with fewer treatments options and a focus on palliative care[2]. Micrometastasis, tiny undetectable tumors that are precursor to secondary tumors, were found in the bone at the time of diagnosis in approximately 30% of patients[3]. Metastasis to the bone can cause immense pain that decreases the quality of life in patients but is not life-threatening[4]. However, metastasis from the bone to lung lowers the life expectancy of the patient significantly[5]. Therefore, preventing the spread of tumor growth from bone to further tissues is ideal. Diet may play an important role in determining the progression of breast cancer.

Menopause, which is marked by a reduction in ovarian function and a decline in the production of estrogens, is a natural process of aging in women. During menopause, symptoms such as weight gain, hot flashes, night sweats, mood changes, and diminished sexual function[6] may appear as a result. Hormone Replacement Therapy (HRT) was commonly used to treat menopausal symptoms up until 2002, when results from the Women's Health Initiative Study stated that hormone replacement therapy (HRT) in the forms of estrogen or estrogen with progesterone, were associated with a higher incidence of invasive breast cancer [7]. Multiple epidemiological studies[8,9] as well as randomized controlled trials[10,11] have supported the original findings. Due to the increased risk to benefit ratio, use of HRT has decreased dramatically, and women have sought alternative treatments such as botanical supplements, which are thought to improve menopausal symptoms through their phytoestrogenic properties[12].

The phytoestrogens found in botanical supplements are structurally and behaviorally similar to estrogen, although their actions tend to be much weaker compared with estrogen. Estrogens play a crucial role in the female body by regulating menstruation, sexual development,

sexual reproduction, and metabolism, but can also contribute to the progression of breast cancer by binding to either of the estrogen receptors (ERs), ERα or ERβ. ERα is thought to carry out pro-oncogenic actions while ERβ plays an opposing role[13,14]. Botanicals have a higher affinity toward ERβ than ERα, but since the ERs share a large homology, possess the potential to turn on or off genes that regulate cancer growth[15].

Licorice root is a botanical supplement product that is marketed to relieve menopausal symptoms and is consumed by women. Licorice root has long been used for its sweetening and flavoring properties, but is also a botanical containing many bioactive components including triterpenoids, polyphenols, flavonoids and its effects on breast cancer are uncertain. There is evidence that licorice root has phytoestrogenic effects, with multiple components exhibiting estrogenic activity[16]. Isoliquiritigenin, a chalcone, is a major phytoestrogenic component found in licorice root which is in isomeric equilibrium with its flavonone liquiritigenin[17]. Both can bind to ERα and ERβ of various cell types and have displayed a biphasic effect on proliferation of breast cancer cells[18,19].

Studies regarding the role of high dietary fat consumption and breast cancer are inconsistent, but some suggest a positive correlation. Higher incidence of invasive breast cancer was found in women with higher intakes of total fat and saturated fat when compared with women with the lowest intakes of fat[20,21]. Women consuming greater amounts of saturated fat, monounsaturated fat, and total fat before breast cancer diagnosis were also reported to have a higher risk of death[22]. A diet high in dietary fat, along with the use of botanical supplements for menopausal symptoms, may have unintended consequences for those with or at high risk for developing breast cancer. The interactions exerted by a diet high in fat can complicate the situation.

In this study, we evaluated the potential efficacy of licorice root compounds in controlling the progression of metastatic breast cancer in the presence of a high fat diet. Our hypothesis is that licorice root compounds act as weak estrogens and will behave differently in hormone-sensitive tissues when compared with estrogen. We predicted that the supplementation of licorice root compounds will reduce metastasis to the lung when consumed with a control and high fat diet. This study investigated the safety of licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) supplementation with and without high fat diet in a metastatic breast cancer mouse model. Outcomes measured were the influence of licorice root on blood serum metabolite levels, extent of metastatic growth to the lungs, hormone responsive tissues, and liver morphology. We found that LRP and ILQ, but not LRE reduced lung metastasis from the bone and that these effects were abolished when licorice root compounds were provided with a high fat diet. Additionally, none of the licorice root compounds induced growth in uterine or mammary gland tissues.

Methodology

Materials

Heat-Inactivated Fetal Bovine Serum (HI-FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Modified IMEM, Penicillin/Streptomycin, Fungizone and Trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). L-Glutamine was purchased from Sigma Chemical Co. (St Louis, MO). Matrigel^{TM} matrix was purchased from BD Biosciences (San Jose, CA). D-luciferin potassium salt was purchased from Regis Technologies (Morton Grove, IL). Isoflurane was purchased from Baxter Healthcare Corporation (Deerfield, IL).

Cell Culture

Murine 4T1 cells with firefly luciferase gene were originally provided by Dr. David Piwnica-Worms from Washington University (St. Louis, MO). Murine 4T1 cells are a clonal tumor line derived from a spontaneous mammary tumor found in a BALB/cfC3H mouse[23]. Though these cells lack the expression of ER[24,25], their growth can be stimulated by estradiol[26]. When injected into Balb/c mice, 4T1 cells spontaneously metastasize to other organs in a way that is similar to how breast cancer metastasis occurs in humans[27,28]. Cells are cultured in IMEM, supplemented with 10% HI-FBS, 100 unit/mL Penicillin, 100 µL/mL Streptomycin, 1% L-Glutamine, and 0.1% Fungizone in a humidified incubator containing 5% CO_2 at 37°C. Cells were harvested on passage 4 at 80% confluency.

Animal Model

Female Balb/C mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were ovariectomized at 3 weeks old by the vendor and arrived at 5 weeks old. Animals were housed in single cages with standard light-dark cycle (12 hours light and 12 hours dark) and provided *ad libitum* access to food and water for the entire study, which lasted a total of 5 weeks. Mice were injected with 4T1 tumors in the tibia to mimic micrometastatic cancer in the bone after 2 weeks of feeding supplemented diet. Mice are kept under general anesthesia with isoflurane/oxygen gas throughout the surgery. During the surgery, the mouse is placed facing up. The surface of the right tibia is shaved and an incision is made to expose the patellar tendon. A hole is created by inserting a 26-gauge needle into the joint surface of the tibia through the patellar tendon into the bone marrow cavity. A 25- µL Hamilton microsyringe with a 27-gauge needle is used to deliver 1,000 4T1 cells suspended in 2.5 µL Matrigel into the hole. The incision is sealed with tissue adhesive (3M Vetbond) and closed by surgical staple. Banamine (2.3 µg/g body weight) was administered subcutaneously after surgery and at 12 hours after surgery. All

animals were sacrificed 3 weeks after cell injection. All studies were conducted under animal experiment protocols approved by the Institutional Animal Care and Uses Committee (IACUC) at the University of Illinois at Urbana-Champaign.

Diet Composition

The study lasted a total of 5 weeks. After one week to allow for acclimation, mice from each study were randomized into two groups and were fed a standard AIN-93G control diet (C) containing16% kcal from fat (kcal/g) or modified AIN-93G high fat diet (HF) containing 45% kcal from fat in pellet forms until the end of the study. The AIN-93G semi-purified diet was chosen because it fulfills all the nutrient requirements of a mouse[29]. Diets were customized by Harlan Diets in order to match the percentage of kilocalories from macronutrients in the diets as closely as possible (Appendix A). Body weights and food intake were monitored weekly throughout the study. Food intake was calculated by subtracting the total amount of food left after one week from the total amount of food provided at the beginning of each week.

Preparation of Licorice Compounds

After 2 weeks from beginning of the study, C and HF diets were supplemented with licorice root powder (LRP), licorice root extract (LRE), isoliquiritigenin (ILQ) or no compound. These diets were supplied *ad libitum* to animals until sacrifice. Doses were selected based on predicted consumption levels by humans from dietary supplements[30]. Licorice root powder (Glycyrrhiza Glabra species) was prepared from the dried roots of licorice plant. Licorice root extract was then obtained from the methanolic fraction of LRP. LRP, (estimated to contain about 10 mg of liquiritigenin and isoliquiritigenin per 1 g licorice root powder),[31] was incorporated into C or HF diet at 500 g/kg of diet (the maximum volume that could be added without altering diet composition). LRE was incorporated into C or HF diet at 50 g/kg of diet. ILQ was incorporated

into C or HF diet at 5 g/kg of diet. LRP and LRE were provided by University of Mississippi. Isoliquiritigenin was purchased from Changsha Heir Biological-Tech Co, Ltd (China).

Liver, Uterus, and Adipose Weights

Liver, uterus, and adipose weights were recorded at sacrifice. Left liver lobe and adipose tissue was flash-frozen and stored at -80°F. Right liver lobe, uterus, kidneys, spleen, and heart was preserved in formalin. Liver lobe was embedded into paraffin, sectioned onto slides, and stained with Hematoxylin and Eosin (H&E). Liver tissues were then graded using a scale of 0 to 5 for the severity of lesions, where 0= none and 5=severe. The grading was evaluated in blind with no knowledge of treatment groups.

Lung Histopathology

The degree of metastasis was measured by quantification of lung tumor nodules. At sacrifice, lungs were excised, fixed in 10 % formalin for 24 hours and then stored in ethanol. Lungs were examined for surface tumor metastases by 3 individuals and counts were averaged. Any interaction effect between licorice root and high fat diet was determined using two-way ANOVA. Lungs were then embedded in paraffin and sectioned into 5 µm slices for H&E staining. Sectioned lung tissues were scanned and observed using NanoZoomer Digital Pathology System (Hamamatsu Phototonics). Metastatic tumor area was measured as described[32]. Tumor area was presented as percentage of whole lung tissue area per section. All slides were measured in a blind fashion without the knowledge of treatment groups.

Mammary Whole Mount

During sacrifice, mammary gland tissue was excised, spread out on glass slide, and placed immediately into Carnoy's fixative. Staining of whole mammary mounts was carried out per instructions provided by VitroView™ Mammary Gland Whole Mount Stain Kit. Total

numbers of terminal end buds were counted by three individuals, independently and without knowledge of treatment groups.

Bioluminescent Imaging (BLI)

Animals were imaged using a custom made imaging system with dual microchannel plate ICCD camera twice weekly after cell injection for 3 weeks until end of study. 15 mg/mL luciferin was dissolved in PBS and 10 µL luciferin per gram of body weight administered into the animal via IP injection. After three minutes, the animal was anesthetized with isoflurane/oxygen gas and placed into the BLI imaging chamber (Stanford Photonics, Palo Alto, CA). A grey-scale image of the mouse was first recorded. Photon emission was then integrated for 3 minutes using imaging software Piper Control (Stanford Photonics, Palo Alto, CA) and visualized in pseudo-color. Images were processed to visualize location and spread of tumors using Piper Control Software, Image J (NIH, Bethesda, MD) and Photoshop Elements (Adobe, San Jose, CA) by merging grey-scale images and bioluminescent signals. Tumor area and integrated density on bone were analyzed using Image J.

Blood Analysis

Blood was drawn for serum metabolite analysis at sacrifice. A validated LC/MS/MS method with internal standard quantification (d3-daidzein and d4-genistein for LIQ and ILQ, respectively) was used to quantify total conjugated and aglycone forms of LIQ and ILQ (LODs 0.2–6 nM depending on volume analyzed), without and with enzymatic hydrolysis, respectively (n = 8–10 mice).

Statistical Analysis

An interaction effect was detected using two-way ANOVA (p=0.02459). Data was analyzed using one-way ANOVA to compare treatment effects of licorice botanicals within C or HF diets, followed by Hochberg procedure using statistical software R (version 3.1.3).

Results

Serum Concentrations of Liquiritigenin and Isoliquiritigenin

Licorice root compounds were provided to animals for a total of five weeks (two before and three after cell injection.) On average, animals ingested approximately 2-3 g of feed per day and weighed an average of 23 g. This equated to about 5000 mg/kg BW LRP, 500 mg/kg BW LRE and 50 mg/kg BW ILQ per day. The studies were orchestrated to deliver quantities of whole botanical, botanical extract and isolated bioactive compound that contained comparable amounts of ILQ. Serum levels of isolquiritigenin primary metabolites, liquiritigenin and isoliquiritigenin, were found to be comparable in animals receiving LRP, LRE or ILQ (Table 3.1). Because the timing of the last consumption of diets was not known with respect to sacrifice, the single-point estimates contain considerable uncertainty.

Effects of Licorice Botanicals on Metastatic Breast Cancer

There was no significant difference between metastasis observed in C and HF animals. In C animals supplemented with licorice root compounds, LRP and ILQ animals had a significant reduction in the number of tumors on the lung surface, while no significant reduction was found with LRE. (Figure 3.1) In HF animals supplemented with licorice root compounds, no changes were found in metastasis to the lung.

Effects of Licorice Botanicals on Uterus Weight and Mammary Tissue

In C animals supplemented with LRE, uterus weight was significantly increased. Uterus weight was not altered in all other animals receiving licorice root compounds (Figure 3.2) No

changes in mammary gland characteristics were seen in animals on HF diet when compared with those on C diet (Figure 3.3). Feeding of licorice root compounds did not impact the proliferation of terminal end buds in the mammary gland within C groups or HF groups (Figure 3.4).

Effects of Licorice Botanicals on Body Weight and Energy Intake

Weight gain in animals fed C and HF diet were comparable and did not significantly differ throughout the study. Addition of licorice root compounds did not alter food intake of C or HF animals (Figures 3.5 and 3.6). Although food intake was higher in HF animals, average energy intake between animals fed C and HF diet were the same (Figures 3.7 and 3.8). HF feeding had no effect on gonadal adipose tissue deposits when compared with C diet. There were no changes observed in gonadal adipose tissue weights in animals fed licorice root compounds (Figure 3.9).

Evaluation of Liver Morphology After Supplementation with Licorice Botanicals

To assess any changes in organ morphology after licorice consumption, liver weight was collected at sacrifice. There were no differences in liver weight between animals fed C or HF diet. Liver weight was increased in mice consuming LRP ($p<0.01$) but was unaltered with supplementation with LRE and ILQ (Figure 3.10). Livers were graded for degree of diet-induced hepatocellular hypertrophy. Minimal to moderate hepatocellular hypertrophy was observed in livers of all animals fed LRP. Minimal to mild hepatocellular hypertrophy was observed in livers of animals fed LRE. Hepatocellular hypertrophy was not found in animals that did not receive licorice root compounds, except one. Results are summarized as averaged scores (Figure 3.11).

Discussion

The goal of this study was to determine the effects of licorice root supplementation on metastasis from bone to lung in a preclinical late-stage BC model within a C or HF diet. We

discovered that LRP and ILQ at the levels we provided reduced lung metastasis when supplemented with a C diet but had no effect on lung metastasis when supplemented with a HF diet. Licorice root botanicals did not increase metastatic growth in our animals. There were also no effects found in uterine tissue and mammary gland tissue, implying that licorice root compounds produced an estrogenic response different from that of estrogen.

Previous literature has shown potential cancer-fighting benefits of licorice root in various forms, including the reduction of breast tumor growth[33,34]. Our study found that the consumption of LRP or ILQ in a C diet significantly reduced metastasis from bone to lung in our animal model. LRE had no significant effect on lung metastasis when compared to the control diet. Licorice root treatment has been shown to decrease factors associated with metastasis, such as VEGF and matrix metalloproteinases (MMPs) 2 and 9, as well as decrease the lung metastasis in mice inoculated with renal carcinoma cells and human breast cancer cells[35-38]. Our results with licorice root powder and isoliquritigenin are consistent with these studies. Though LRE did now show any significant effects, there was a trend toward reduction. The same consumption of LRP, LRE and ILQ in a HF diet had no effect on the extent of metastasis to the lung.

Mammary gland proliferation was measured by counting the number of terminal end buds. Consumption of licorice root containing diets did not have any impact on the proliferation of terminal end buds. In addition, no impact was seen on the uterus weight, suggesting that LRP, LRE and ILQ did not have a proliferative effect on these reproductive tissues in animals. Feeding of licorice root compounds was determined not to have an effect on uterus weight or mammary gland and produced no changes in gene expression in these tissues in a similar study using C57 mice[39]. Given what we know about the estrogenicity of licorice root compounds[19,40], these results suggests that licorice root may behave similarly to a selective estrogen receptor modulator

(SERM), in that it can exhibit agonistic or antagonistic activities when binding to estrogen receptor depending on the target tissue. Another study supports this idea by illustrating that components found in licorice root extract in addition to isoliquiritigenin and liquiritigenin were potent estrogen agonists or partial estrogen antagonists[41]. However, licorice root contains multiple bioactive compounds in addition to isoliquiritigenin that could be acting through various mechanisms of action. In order to confirm whether or not these physiological changes are solely due to binding of estrogen receptor, studies combining the administration of these compounds with an antiestrogen could be warranted.

Blood serum metabolite levels showed that levels of liquiritigenin/isoliquiritigenin in the licorice root study were variable. Since diets were provided *ad libitum*, feeding times of animals were unregulated and the timing of last meal consumption was unknown. Thus, blood serum metabolite levels may not have been accurately reflected. Future studies should consider implementation of a feeding regimen providing specified amounts of diet at fixed times to avoid inconsistencies.

Overall, body weight gain did not differ between C and HF groups. Compared with levels of fat used in high fat cancer models of other studies, the amount of fat in our C groups was higher than the amounts used in others control diets, while animals in our HF groups received lower amounts of fat. In earlier studies, animal were fed control diets containing 10-13.5% kcal from fat and high fat diets containing 45-60% kcal from fat, with a feeding period of 16 weeks or longer[42-44]. We chose to use a diet containing 45% kcal from fat to parallel levels of fat attainable in humans (average consumption in U.S women is estimated to be ~33%)[45], with a feeding period of 5 weeks. Balb/c mice are postulated to be highly obese-resistant due to increased

thermogenic capacity and efficient food intake regulation[46]. This model allowed us to isolate the effects of a high-fat diet without any confounding variables that might arise from obesity.

Liver weights were significantly increased in the animals receiving LRP, and examination of liver tissue showed mild to moderate hepatocellular hypertrophy that is typical of liver adaptation to xenobiotics. The changes in the liver were not considered to be permanent, but long-term consumption of licorice in predisposed populations has been known to cause adverse effects such as edema, hypertension and hypokalemia[47]. These side effects have only been associated with glycyrrhizin, which is the major flavor component found in licorice root. LRE and ILQ, which do not contain glycyrrhizin, did not appear to have any significant effects at the levels consumed in our studies. While it was determined that consumption of 100 mg/day glycyrrhizin would not cause adverse effects in most adult humans[30], it would be advisable in future long-term studies to limit or remove glycyrrhizin content from licorice components in order to obtain beneficial effects of licorice root without risk of toxicity from glycyrrhizin. Minimal changes were seen in kidney and spleens of animals receiving licorice root compounds compared with C or HF animals. Further examination of sectioned kidney and spleen tissues did not produce any evidence of structural abnormalities (Appendix A).

In conclusion, licorice root did not contribute to the progression of late-stage metastatic breast cancer in our animal model. While licorice root had no effect on reproductive tissues, licorice root powder and isoliquiritigenin reduced the degree of metastasis within a control diet. The beneficial effects of licorice root on breast cancer metastasis were not seen with a high fat diet. Continued research of licorice root and the mechanisms behind these findings would help gain insight as to appropriate consumption of licorice root in women with breast cancer and indications for consumption with other components in the diet that may cause interactions.

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Tables

Table 3.1. Serum metabolites of licorice compounds. Blood levels of liquiritigenin (LIQ) and isoliquiritigenin (ILQ), as total and aglycones, in animals on control diets (C) or high fat diets (HF) containing licorice root powder (LRP), licorice root extract (LRE), or pure isoliquiritigenin (ILQ).

Table 3.1

Dose Group	LIQ-Total (µM)	LIQ-Aglycone (µM)	% Aglycone	LIQ/ILQ Ratio	ILQ-Total (µM)	ILQ-Aglycone (µM)	% Aglycone
C	<LOD[a]	<LOD	--	--	<LOD	<LOD	--
C-LRP	3.8	0.0034	0.1	8	0.46	0.0041	0.9
C-LRE	1.9	0.0175	0.9	11	0.17	0.0014	0.8
C-ILQ	0.037	<LOD	--	0.3	0.13	0.0083	6.4
HF	0.014	<LOD	--	--	<LOD	<LOD	--
HF-LRP	2.9	0.0054	0.2	11	0.27	0.0023	0.9
HF-LRE	1.4	0.0036	0.3	16	0.09	0.0008	0.9
HF-ILQ	0.055	0.0004	0.7	0.3	0.18	0.013	7.1

[a]<LOD=below level of detection

Figures

Figures 3.1. Lung Tumor Metastasis. Surface tumors found on lungs of Balb/c mice after feeding licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. N=10. p <0.05, N=10. Groups sharing a letter are not significantly different at the 5% level.

Figure 3.1

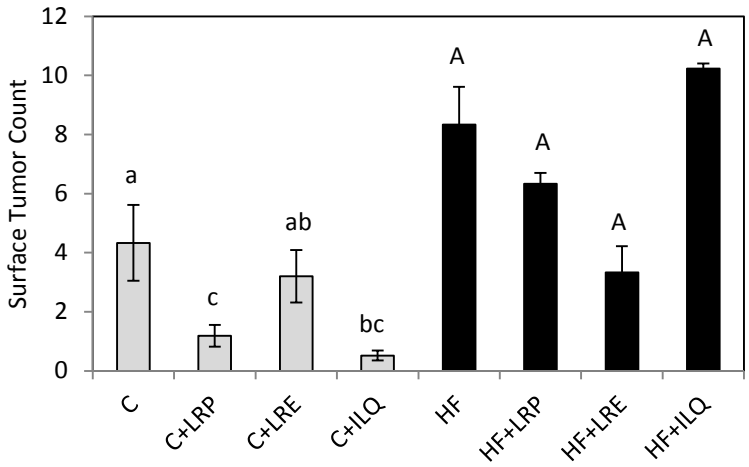
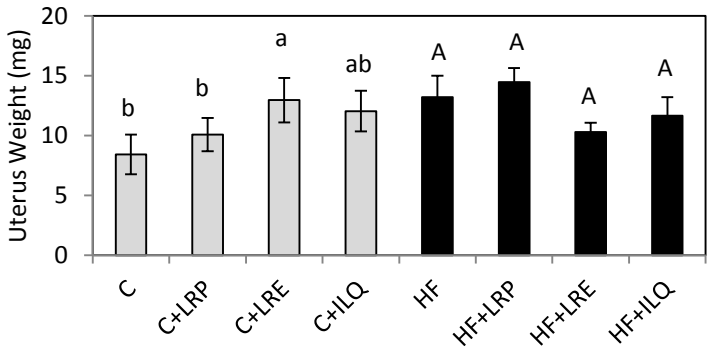


Figure 3.2. Uterus Weight Measurements. Changes in uterus weight after 4 weeks of feeding licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. p<0.05, N=10. Groups sharing a letter are not significantly different at the 5% level.

Figure 3.2



Figures 3.3 and 3.4. Mammary Gland Proliferation. Representative images of mammary glands found in mice after 4 weeks of feeding licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a Control (C) or High Fat (HF) diet (**Figure 3.3**). Terminal end bud numbers are expressed as means +/- SEM. $p>0.05$, $n=10$. Groups sharing a letter are not significantly different at the 5% level. (**Figure 3.4**)

Figure 3.3

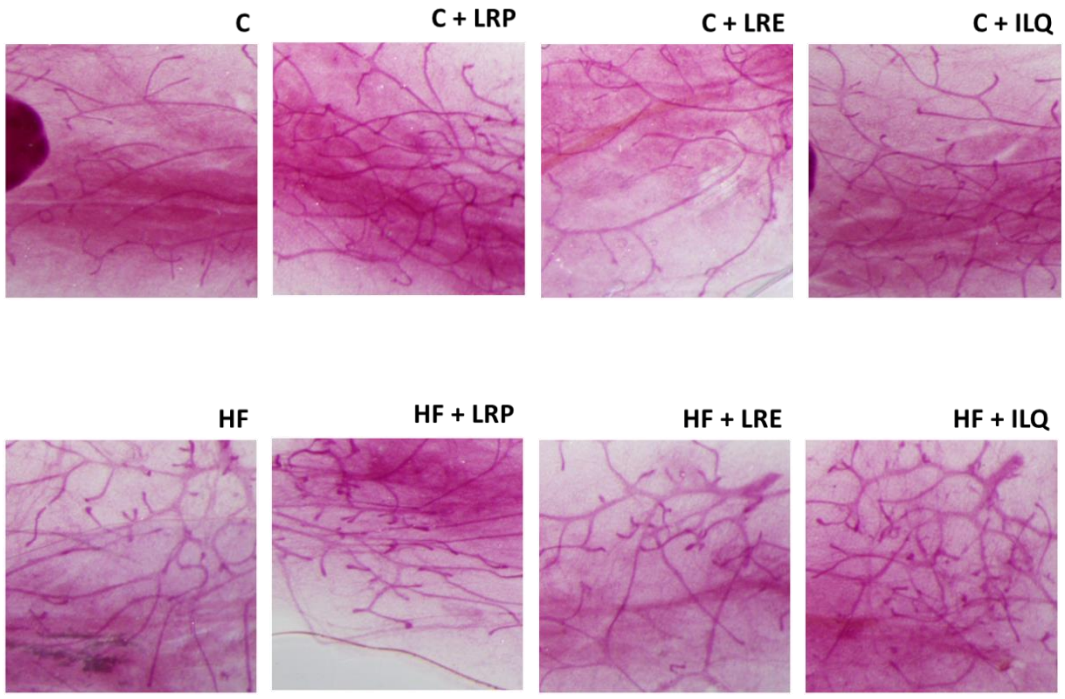


Figure 3.4

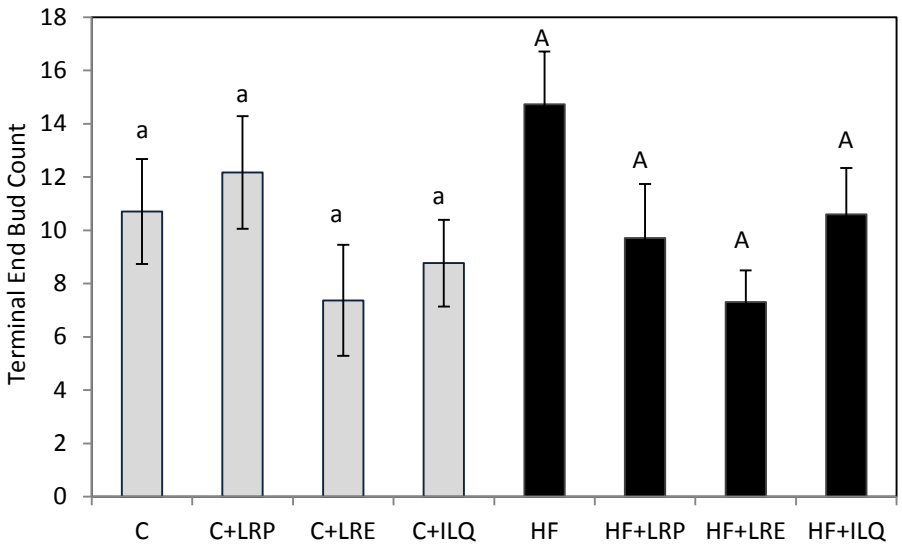


Figure 3.5, 3.6, 3.7, 3.8. Body Weight and Food Intake. Weekly food intake of Balb/c mice fed a Control (C, **Figure 3.5**) or High Fat (HF, **Figure 3.6**) diet and average energy intake (kcal) per day of Balb/c mice fed a C (**Figure 3.7**) or HF (**Figure 3.8**) diet. Results are reported as means +/- SEM. p>0.05, N=10.

Figure 3.5

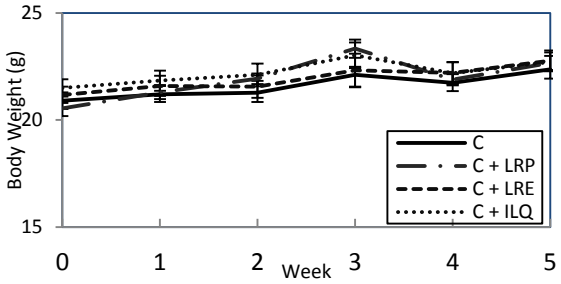
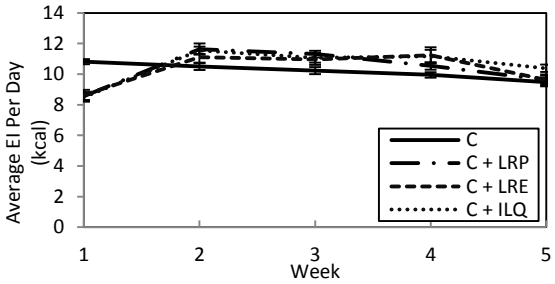


Figure 3.7



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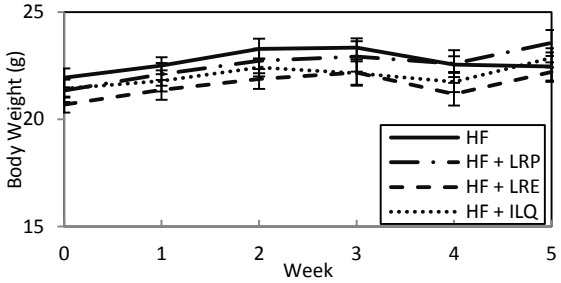


Figure 3.8

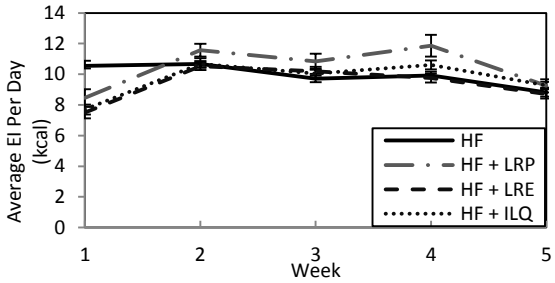


Figure 3.9. Gonadal Adipose Tissue Measurements. Changes in gonadal adipose tissue weight (g) after 4 weeks of feeding feeding licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. p>0.05, N=10. Groups sharing a letter are not significantly different at the 5% level.

Figure 3.9

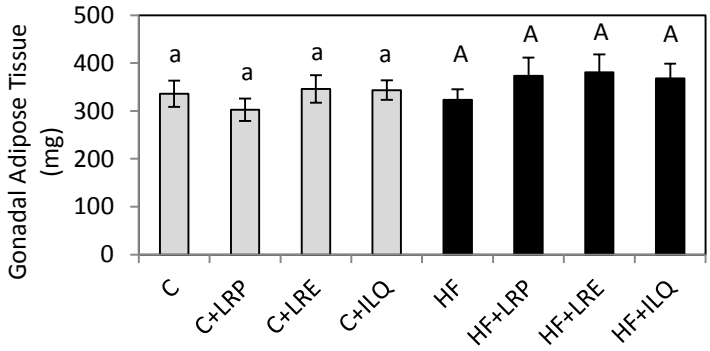


Figure 3.10. Liver Weight Measurements. Changes in liver weight (g) after 4 weeks of feeding licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. $p<0.05$, N=10. Groups sharing a letter are not significantly different at the 5% level.

Figure 3.10

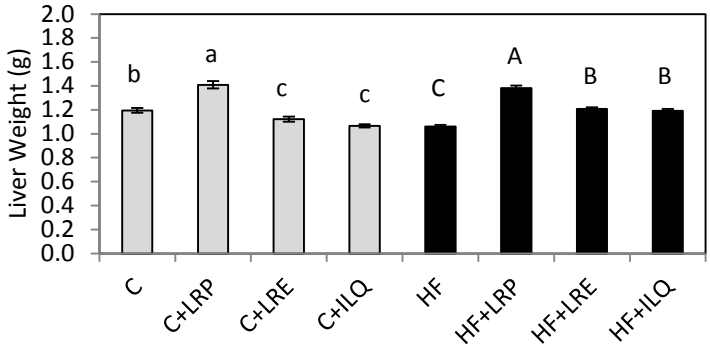
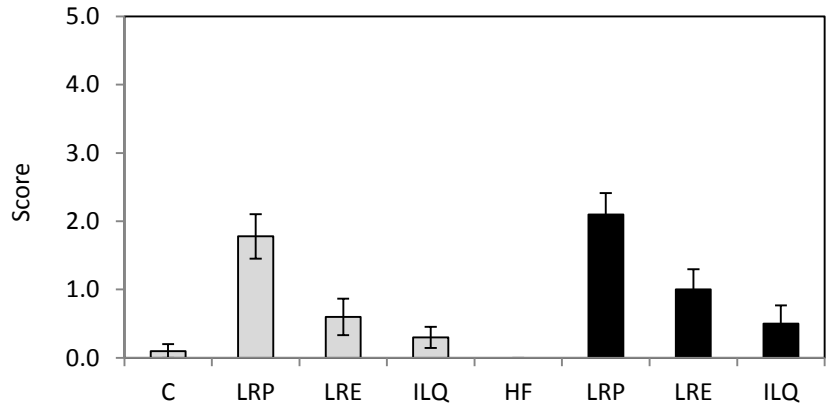


Figure 3.11. Hepatic Tissue Sectioning. Summary of graded hepatocellular hypertrophy (0=none to 5=severe) of livers from mice fed licorice root powder (LRP), licorice root extract (LRE), and isoliquiritigenin (ILQ) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. N=10

Figure 3.11



CHAPTER 4: EPIMEDIUM STUDY

Abstract

Metastasis is a leading cause of death in breast-cancer (BC) related deaths. While the mechanisms that induce metastasis in breast cancer patients remain unclear, effects of diets have been implicated as a critical factor. Botanicals from the genus Epimedium have been marketed toward women for relief of menopausal symptoms, and have been shown to proffer multiple health benefits, including prohibitory growth effects in cancer. Epimedium has been postulated to have estrogenic properties, which can modulate transcription of genes that promote or repress tumor growth. Studies have also suggested that higher consumption of dietary fat is related to breast cancer risk. The safety and efficacy of epimedium in promoting health among postmenopausal women who are at high risk of developing BC remains undetermined. Our group has characterized a preclinical model with metastatic 4T1 murine cells implanted into the tibial bone of Balb/C mice. This model is also responsive to changes in energy balance. Using this preclinical model, we provided amounts of epimedium powder (EP), epimedium extract (EE) and icariin (II) that would result in blood levels in mice similar to those found in humans. Our goal was to assess the potential health risk and/or benefit of epimedium using this model by examining the effect of epimedium on BC growth and metastasis in conjunction with a diet high in fat that is comparable to levels consumed in the general population. After 4 weeks of dietary supplementation, we found that EP, EE, and II did not stimulate metastatic growth within a control diet, while supplementation with EE (but not EP or II) reduced the amount of metastasis within a high fat diet. In addition, dietary EP and EE, but not II, stimulated uterine and mammary gland tissue growth. Our results indicate that epimedium compounds induce

differential estrogenic responses in various tissues and may act as selective estrogen receptor modulators (SERMs).

Introduction

Breast cancer (BC) encompasses the largest percentage of cancers in women worldwide and is the most significant contributor to cancer related mortality[1]. Though breast cancer mortality rates have declined in the last ten years, there are still 40,000 American deaths due to breast cancer each year. Most of these deaths are due to cancer metastasis. This is the spread of cancer from the primary site to other areas of the body. It is estimated that 20-30% of all early-stage breast cancers will become metastatic[2]. There is a strong likelihood of recurrence in distal tissues in women who have been successfully treated for a primary tumor as well. Bone metastasis occurs in 70% of patients with late-stage BC[3], with micrometastasis in the bone marrow detected in 30.6% of women with invasive breast cancer before any diagnosis of metastatic breast cancer[4]. Patients with metastasis are estimated to have a 5-year survival rate of 26%[5]. There are currently no established standards of care for metastatic breast cancer. Therapeutic measures focus on disease control, palliative care for symptoms, and to maintaining quality of life[6].

The causes of tumor progression remain unclear, but dietary factors such as fat consumption may be a large contributor. Although the relationship between high-fat diets and breast cancer is debatable, multiple epidemiological studies implicate a role for high dietary fat consumption on breast cancer prognosis. Thiebaut et al. reported an 11% higher incidence of invasive breast cancer in women with median intake of 40.1% energy from total fat than women with median intake of 20.3% energy from total fat[7]. Sieri et al found a significantly greater risk

of developing breast cancer in women with a median saturated fat intake of 45% energy from saturated fat than with a median intake of 16.2% energy from saturated fat [8].

Complementary and alternative medicine (CAM) has become increasingly prevalent in the U.S and has an estimated expenditure of $34.4 billion each year[9]. People with chronic disease are more likely to be CAM users than those without illness[10]. As high as 86% of breast cancer patients or survivors have been reported to use at least one form of CAM after diagnosis[11-13]. Breast cancer survivors who were CAM users and were at least one year beyond active medical treatment reported initiating or changing CAM activity due to their cancer diagnosis[14]. Wanchai reported that a large portion of patients are using biological-based practices such as herbs, vitamins and food[15]. Of particular concern are the use of botanical supplements, which contain phytoestrogens with varying levels of estrogenicity.

Estrogen exposure can be a major contributor to breast cancer by stimulation of growth through estrogen signaling[16]. Estrogen signaling occurs through the binding of estrogen receptor (ER) by estrogen. There are predominantly two types of estrogen receptor, ERα and ERβ, which are found in many tissues of the body, like uterus, ovary, mammary gland, prostate, lung and brain. ERα is thought to carry out pro-oncogenic actions while ERβ plays an opposing role[17,18]. Phytoestrogens have similar structures to estrogen and tend to have a higher affinity toward ERβ than ERα [19]. Due to the large homology of the ERs (~59%), phytoestrogens are still able to bind to ERα and therefore have the capability to stimulate cell growth.

Plants of the genus Epimedium, also known as “barrenwort”, “horny goat weed” and “ying yang huo”, are marketed as a supplement for relief menopausal symptoms. It has a history for use in traditional Chinese medicine as an aphrodisiac and has also been used to treat a range of ailments such as impotence, hyperdiuresis, osteoporosis, menopause syndrome, rheumatic

arthritis, and hypertension[20]. Epimedium contains several flavonoids and lignans, many of which have been characterized to have estrogenic activities. The flavonoid Icariin is the major estrogenic bioactive present in many commonly used species of Epimedium, including *Epimedium sagittatum, Epimedium brevicornum,* and *Epimedium koreanum*. Studies have shown that epimedium possesses numerous benefits, including phytoestrogenic[21,22], osteoporotic, cardialogical[23,24], and neuroprotective[25,26] properties. However, there is a lack of conclusive evidence on the safety and efficacy of epimedium pertaining to breast cancer. Icaritin and desmethylcaritin, metabolites of icariin, have been shown to stimulate breast cancer cell proliferation[27,28] while epimedium extract, icariin and icaritin have been shown to reduce the growth of breast tumors in a mouse model[29,30]. More studies are needed to determine the effects of epimedium and its relationship with breast cancer growth.

Our goal was to determine the safety of epimedium consumption in menopausal women who are at risk for BC. Our hypothesis was that epimedium compounds are weak estrogens that will act similarly to licorice root. We predicted that supplementation of epimedium compounds will be able to reduce the number of metastatic lung nodules and that a high fat diet would negate or alter those effects. To test our hypothesis, we investigated the effects of epidemium powder (EP), epimedium extract (EE), and icariin (II) on metastasis of 4T1 murine breast cancer from bone to lung in a preclinical ovariectomized Balb/C model. Supplementation with EP, EE, and II had no effects on metastasis with a control diet, while supplementation with EE (but not EP or II) was found to reduce the amount of metastasis with a high fat diet.

Methodology

Materials

Heat-Inactivated Fetal Bovine Serum (HI-FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Modified IMEM, Penicillin/Streptomycin, Fungizone and Trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). L-Glutamine was purchased from Sigma Chemical Co. (St Louis, MO). Matrigel^{TM} matrix was purchased from BD Biosciences (San Jose, CA). D-luciferin potassium salt was purchased from Regis Technologies (Morton Grove, IL). Isoflurane was purchased from Baxter Healthcare Corporation (Deerfield, IL).

Cell Culture

Murine 4T1 cells with firefly luciferase gene were originally provided by Dr. David Piwnica-Worms from Washington University (St. Louis, MO). Murine 4T1 cells are a clonal tumor line derived from a spontaneous mammary tumor found in a BALB/cfC3H mouse[31]. Though these cells lack the expression of ER[32,33], their growth can be stimulated by estradiol[34]. When injected into Balb/c mice, 4T1 cells spontaneously metastasize to other organs in a way that is similar to how breast cancer metastasis occurs in humans[35,36]. Cells are cultured in IMEM, supplemented with 10% HI-FBS, 100 unit/mL Penicillin, 100 µL/mL Streptomycin, 1% L-Glutamine, and 0.1% Fungizone in a humidified incubator containing 5% CO_2 at 37°C. Cells were harvested on passage 4 at 80% confluency.

Animal Model

Female Balb/C mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were ovariectomized at 3 weeks old by the vendor and arrived at 5 weeks old. Animals were housed in single cages with standard light-dark cycle (12 hours light and 12 hours dark) and provided *ad libitum* access to food and water. Mice were injected with 4T1 tumors in the tibia to mimic micrometastatic cancer in the bone after 2 weeks of feeding supplemented diet (after 16 weeks from the beginning of the study.) Mice are kept under general anesthesia with

isoflurane/oxygen gas throughout the surgery. During the surgery, the mouse is placed facing up. The surface of the right tibia is shaved and an incision is made to expose the patellar tendon. A hole is created by inserting a 26-gauge needle into the joint surface of the tibia through the patellar tendon into the bone marrow cavity. A 25- μL Hamilton microsyringe with a 27-gauge needle is used to deliver 1000 4T1 cells suspended in 2.5 μL Matrigel into the hole. The incision is sealed with tissue adhesive (3M Vetbond) and closed by surgical staple. Banamine (2.3 μg/g body weight) was administered subcutaneously after surgery and at 12 hours after surgery. All animals were sacrificed 3 weeks after cell injection. All studies were conducted under animal experiment protocols approved by the Institutional Animal Care and Uses Committee (IACUC) at the University of Illinois at Urbana-Champaign.

Diet Composition

The study lasted a total of 19 weeks. After one week to allow for acclimation, mice from each study were randomized into two groups and were fed a standard AIN-93G control diet (C) containing16% kcal from fat (kcal/g) or modified AIN-93G high fat diet (HF) containing 45% kcal from fat in pellet forms until the end of the study. The AIN-93G semi-purified diet was chosen because it fulfills all the nutrient requirements of a mouse[37]. Diets were customized by Harlan Diets in order to match the percentage of macronutrients in the diets as closely as possible (Appendix A). Body weights and food intake were monitored weekly throughout the study.

Preparation of Epimedium Compounds

Dietary treatments with botanicals were provided for a total of 4 weeks. After 14 weeks from beginning of the study, C and HF diets were supplemented with epimedium powder (EP), epimedium extract (EE), icariin (II) or no compound, which were supplied *ad libitum* to animals until sacrifice. Epimedium powder was prepared from the dried leaves of epimedium plant.

Epimedium extract (estimated to contain 10% of the icariin from epimedium powder) was then obtained from the methanolic fraction of epimedium powder. EP was incorporated into C or HF diet at 50,000 mg/kg of diet (the maximum volume that could be added without altering diet composition). EE was incorporated into C or HF diet at 5,000 mg/kg of diet. Icariin was incorporated into C or HF diet at 500 mg/kg of diet. EP and EE were provided by University of Mississippi. II was purchased from Changsha Heir Biological-Tech Co, Ltd (China).

Liver, Uterus, and Adipose Weights

Liver, uterus, and adipose weights were recorded at sacrifice. Left liver lobe and adipose tissue was flash-frozen and stored at -80°F. Right liver lobe, uterus, kidneys, spleen, and heart was preserved in formalin.

Lung Histopathology

The degree of metastasis was measured by quantification of lung tumor nodules. At sacrifice, lungs were excised, fixed in 10 % formalin for 24 hours and then stored in ethanol. Lungs were examined for surface tumor metastases by 3 individuals and counts were averaged.

Mammary Whole Mount

During sacrifice, mammary gland tissue was excised, spread out on glass slide, and placed immediately into Carnoy's fixative. Staining of whole mammary mounts was carried out per instructions provided by VitroView^{TM} Mammary Gland Whole Mount Stain Kit. Total numbers of terminal end buds were counted by three individuals, independently and without knowledge of treatment groups.

Bioluminescent Imaging (BLI)

Animals were imaged using a custom made imaging system with dual microchannel plate ICCD camera twice weekly after cell injection for 3 weeks until end of study. 15 mg/mL

luciferin was dissolved in PBS and 10 µL luciferin per gram of body weight administered into the animal via IP injection. After three minutes, the animal was anesthetized with isoflurane/oxygen gas and placed into the BLI imaging chamber (Stanford Photonics, Palo Alto, CA). A grey-scale image of the mouse was first recorded. Photon emission was then integrated for 3 minutes using imaging software Piper Control (Stanford Photonics, Palo Alto, CA) and visualized in pseudo-color. Images were processed to visualize location and spread of tumors using Piper Control Software, Image J (NIH, Bethesda, MD) and Photoshop Elements (Adobe, San Jose, CA) by merging grey-scale images and bioluminescent signals. Tumor area and integrated density on bone were analyzed using Image J.

Blood Analysis

Blood was drawn to conduct analyses of epimedium metabolites at sacrifice. A validated LC/MS/MS method with internal standard quantification (d3-daidzein and d4-genistein) was used to quantify total II (icariin), ICT (icaritin) and DICT (desmethylicaritin), with n = 16–18 mice.

Statistics

Data was analyzed using two-way ANOVA to compare treatment effects of epimedium botanicals within C and HF diets, using R (version 3.1.3). Tumor nodules counts were analyzed using one-way Poisson regression model adjusted for overdispersion.

Results

Blood Serum Metabolites

Serum levels of icariin primary metabolites, icaritin and desmethylicaritin were found to be similar in animals consuming EP, EE or II with C or HF diets (Table 4.1). Levels of ICT and DICT appear to be twice as high in animals fed EE compared with EP diet. Because the timing

of the last consumption of diets was not known with respect to sacrifice, the single-point estimates contain considerable uncertainty.

Evaluation of Hormone Responsive Tissues

There were no high fat effects detected on uterine weight between animals fed C or HF diet. Supplementation with EP and EE, but not II, in both C and HF diets resulted in heavier uterine weights in mice (Figure 4.1). Epimedium compounds also produced changes visible to the naked eye of mammary glands in mice. Thicker uterine lining and extensive branching was observed (Figure 4.2). Increased numbers of tumor end buds were found in mammary glands of animals fed EP and EE, but not in II (Figure 4.3).

Evaluation of Lung Metastasis

No differences in lung metastasis were found in mice supplemented with epimedium compounds when compared with those consuming C diet. Mice supplemented with EP in a HF diet showed significant reductions in the number of surface tumors found on the lung using one-way Poisson regression to adjust for overdispersion (Figure 4.4).

High Fat Animal Model and Weight Gain

Body weights did not differ between animals in the C or HF diet groups. Supplementation with epimedium compounds did not have an effect on weight gain in C or HF groups (Figures 4.5 and 4.6). Average energy intake was comparable across all groups throughout the study (Figure 4.7 and 4.8). Mice supplemented with epimedium extract (EE) and fed either a C or HF diet had lower gonadal adipose tissue weights (Figure 4.9). Supplementation with epimedium powder (EP) or icariin (II) did not produce any weight changes in mesenteric, peri-renal or brown adipose tissues. (Appendix B).

Evaluation of Organ Weights

There was no impact on liver weights of mice supplemented with epimedium compounds in either C or HF mice (Appendix B). There was an increase in kidney weights of mice supplemented with EP ($p<0.05$), but not EE or II (Figure 4.10). Spleen weights were significantly decreased in kidney weights of mice supplemented with II, but not EP or EE (Figure 4.11).

Discussion

Epimedium and its constituents have been shown to alter cell cycle differentiation in tumor cells and in some cases to stimulate or inhibit cell proliferation, but it is still unclear whether epimedium has stimulatory or inhibitory effects on *in vivo* cancer growth. We found that supplementation of epimedium compounds with C diet produced no changes in tumor metastasis to the lung when compared to C. Epimedium compounds supplemented with HF diet showed a trend in decreasing lung tumors, but due to high variability within the HF group, only EP was statistically significant. Blood serum samples showed that icaritin (ICT) and desmethylicaritin (DICT), metabolites of icariin, were present in very low amounts at pM/μL levels, while levels of icariin were almost nonexistent. This shows that the majority of icariin was metabolized to its metabolites, although the levels of icariin delivered to the animal were most likely low to begin with.

To our knowledge, this study is the first to look at the effects of icariin supplementation on tumor metastasis of breast cancer in a live animal. Animal studies have mainly looked at the anti-cancer potentials of icaritin, which has been shown to suppress growth of cancer cells in a dose-dependent and time-dependent manner, as well as decrease tumor growth in 4T1 breast tumors[38]. Growth of human renal carcinoma xenografts in immunodeficient mice were also decreased with icaritin treatment[39]. Many groups have found that treatment with icariin alone and

in combination with other treatments sensitizes cancer cells and promotes cell death; however, these have not been translated into animal studies. Icariin in combination with gemcitabine, 5-fluorouracil, and radiation therapy has led to decreased cell proliferation and increased apoptosis in colorectal cancer cells through the suppression of NF-KB[40-42]. Other bioactive components found in epimedium besides icariin have shown potential for slowing metastasis. Bauhuoside I was found to downregulate CXCR4, a key mediator of tumor metastasis, in cervical and breast cancer cells[43]. Icariside II decreased cell migration, invasion and epithelial-mesenchymal transition of lung cancer cells, as well as decreased the lung metastasis of nude mice[44]. It is likely that the reductive effects of EP are due to the combined action of several bioactive components which are not present in EE or II.

We found effects from epimedium on two estrogen responsive tissues. Epimedium had a similar growth-promoting effect in both uterine tissue and mammary gland tissue. These actions corresponded with responses in the presence of estradiol. Because our animals were ovariectomized and were not producing any endogenous estrogens, all changes were attributed to treatment effects. Epimedium and icariin have exhibited differential effects at low and high concentrations. Administration of epimedium flavonoids were reported to increase uterine weight at levels of 500 mg/kg of feed and had no effect on uterine weight at higher levels (1500 and 5000 mg/kg feed)[29]. Icariin also increased uterine weight at levels of 10 and 20 mg/kg BW per day[22], while another group found no changes to uterine weight with icariin administration at 110 mg/kg BW[45]. The levels of EP and EE provided in our study at 50,000 and 5,000 mg/kg of feed was much higher than those used in previous studies.

The current study included dietary fat as an independent variable due to the higher consumptions of fat in the average American woman. While the animals in our high fat groups

did not gain a significant amount of weight compared with the control groups, a prior study using the Balb/c athymic nude mouse strain led to altered physiology such as a decrease in splenic NK cells, changes in tumor gene expression involved with cell cycle regulation, overexpression of tumor mRNA glycolytic enzymes, and higher vascular endothelial growth receptor 2 (VEGFR-2) in tumor despite a lack of change in body weight[46]. Animals from other studies had significantly greater body weights when fed a high fat diet compared with control diet, which successfully induced an inflammatory state that resulted in increased tumor growth and/or metastasis. These studies used control diets containing 10-13.5% kcal from fat and high fat diets containing 45-60% kcal from fat, with a feeding period of 16 weeks or longer[47,48]. Our studies used a control diet containing 18% kcal from fat and a high fat diet containing 45% kcal from fat to closer match a level of fat attainable in humans (average consumption is estimated to be ~33% in U.S women)[49], with a feeding period of 16 weeks before cell injection. The Balb/c mouse strain has increased thermogenic capacity and improved fat catabolism, thus making them very effective at regulating food intake[50]. Because Balb/c mouse is known to be highly obese-resistant, we are able to investigate a high-fat model without any confounding variables that might arise with obesity.

Herba epimidii has been shown to produce adverse effects at higher levels when consumed for long periods of time, but most adverse effects have been reported in a specific subset of patients or animals[51]. Because our animals were relatively healthy and received epimedium supplementation for only four weeks, it was unlikely that there would be any toxicity or adverse incidences. In this study, we did not observe any toxic effects from Herba Epimidii. Animals maintained steady body weight growth rates throughout the study, and no changes in liver weight were seen in animals supplemented with epimedium compounds. Animals receiving

EP had increases in kidney weight that were significant. Spleen weights in animals supplemented with II were significantly lower. Although these parameters were significant, these changes do not necessarily indicate toxicity.

Conclusion

The aim of this study was to examine the safety of consuming epimedium compounds as a supplement in a population at risk for developing breast cancer and to assess the impact of consuming botanicals on breast cancer metastasis. We accomplished this by supplementing EP, EE, and II with a C or HF diet in a metastatic mouse model that follows a metastatic pattern from the bone to lung. We found that EP and EE produced significant changes in the uterus and mammary gland indicative of an estrogenic response. Epimedium compounds had no effect on metastasis to the lung when compared with C. EP significantly reduced the extent of metastasis to the lung when compared with HF, while EE and II animals tended to have lower amounts of metastasis but were not significant. Overall, epimedium compounds had no proliferative effects on breast cancer metastasis but induced uterus and mammary gland growth. Our results suggest that the consumption of epimedium is safe in menopausal women who are at risk of developing breast cancer. However, further research is required to confirm these findings and determine safe levels of consumption in humans.

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Tables

Table 4.1. Serum metabolites of epimedium compounds. Blood levels of total icariin (II), desmethylicaritin (DICT), and icaritin (ICT), in animals on control diets (C) or high fat diets (HF) containing epimedium powder (EP), epimedium extract (EE), or pure icariin (II).

Table 4.1

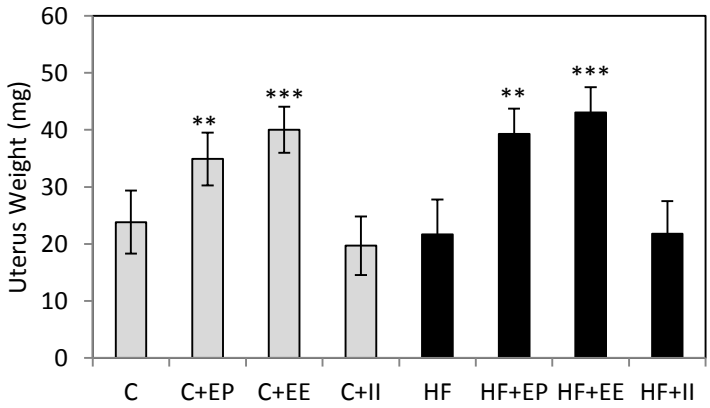
Dose Group	II-Total (pM/µL)	DICT-Total (pM/µL)	ICT-Total (pM/µL)
C	<LOD[a]	<LOD	<LOD
C-EP	0.0007	0.1300	0.0782
C-EE	<LOD	0.2367	0.1865
C-II	0.0010	0.1280	0.0880
HF	<LOD	<LOD	<LOD
HF-EP	0.0004	0.0700	0.0609
HF-EE	0.0004	0.1282	0.1305
HF-II	<LOD	0.0030	0.0159

[a]<LOD=below level of detection

Figures

Figure 4.1. Uterus Weight Measurements. Changes in uterus weight after 4 weeks of feeding epimedium powder (EP), epimedium extract (EE), and icariin (II) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. **p<0.01, ***p<0.001, N=18.

Figure 4.1



Figures 4.2 and 4.3. Mammary Gland Proliferation. Representative images of mammary glands found in mice after 4 weeks of feeding epimedium powder (EP), epimedium extract (EE), and icariin (II) with a Control (C) or High Fat (HF) diet (**Figure 4.2**). Terminal end bud numbers are expressed as means +/- SEM, ***p<0.001,N=18. (**Figure 4.3)**.

Figure 4.2

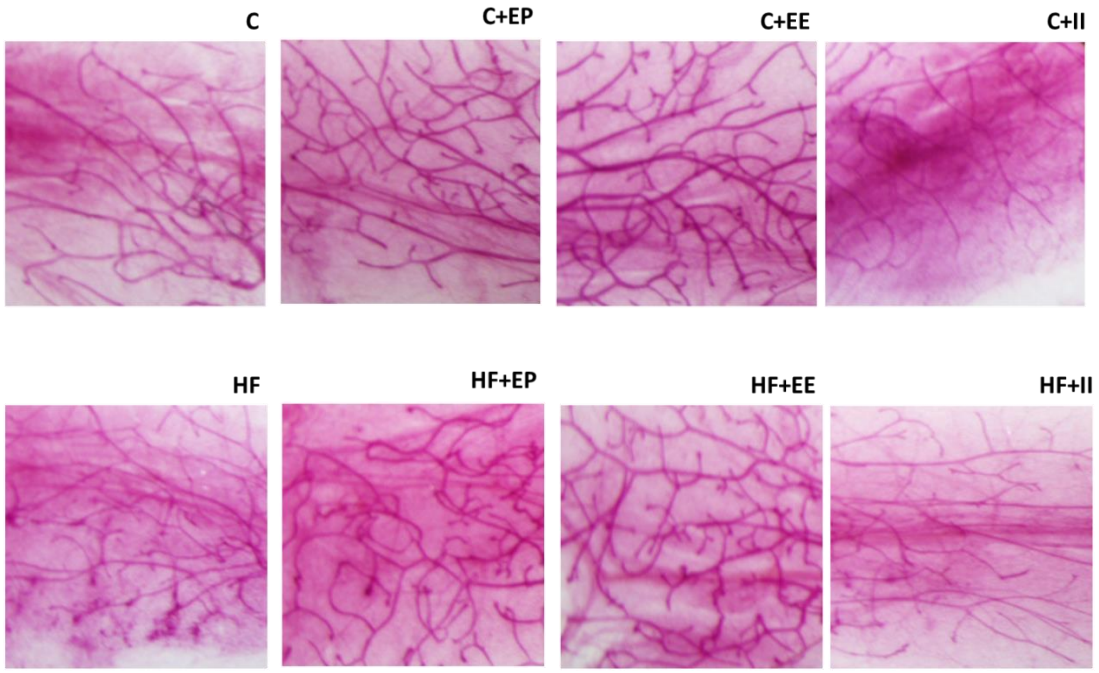


Figure 4.3

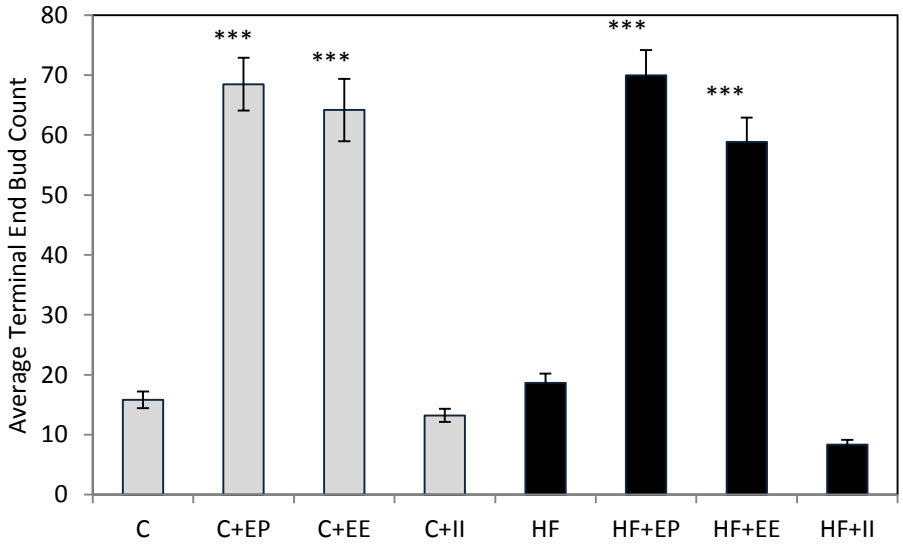
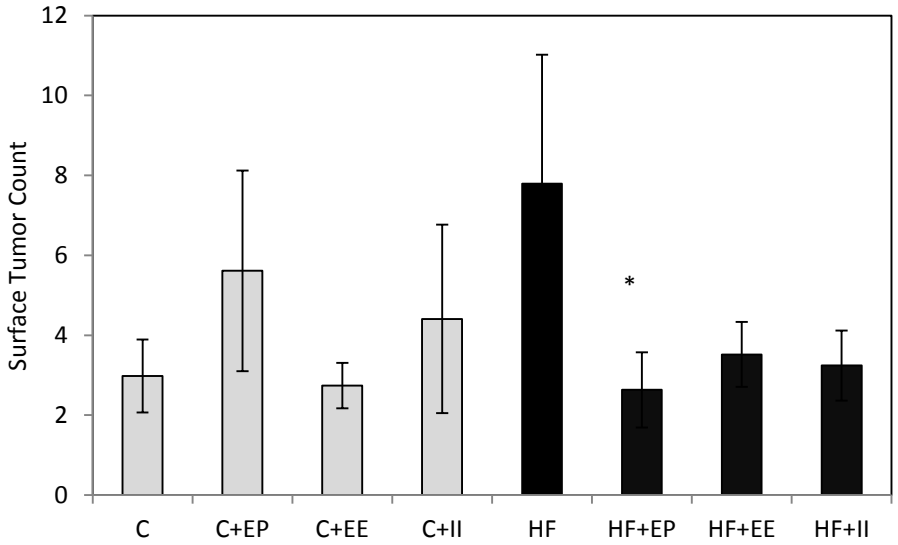


Figure 4.4. Lung Tumor Characterization. Surface tumors found on lungs of Balb/c mice after feeding epimedium powder (EP), epimedium extract (EE), and icariin (II) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. One-way Poisson regression model adjusted for overdispersion. *p<0.05, N=18.

Figure 4.4



Figures 4.5, 4.6, 4.7, and 4.8. Body Weight and Food Intake. Weekly food intake of Balb/c mice fed a Control, C (**Figure 4.5**) or High Fat, HF (**Figure 4.6**) diet and average energy intake (kcal) per day of Balb/c mice fed a Control, C (**Figure 4.7)** or High Fat, HF (**Figure 4.8**) diet. Results are reported as means +/- SEM. p>0.05, N=18.

Figure 4.5

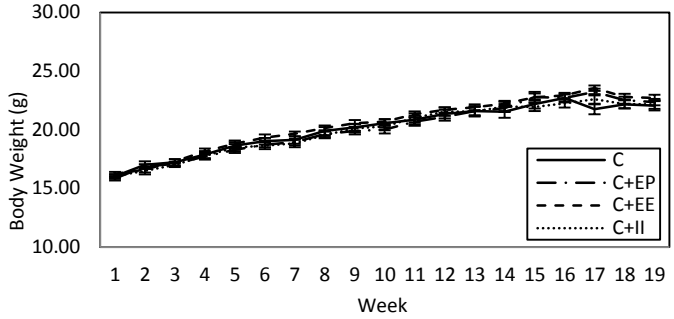


Figure 4.6

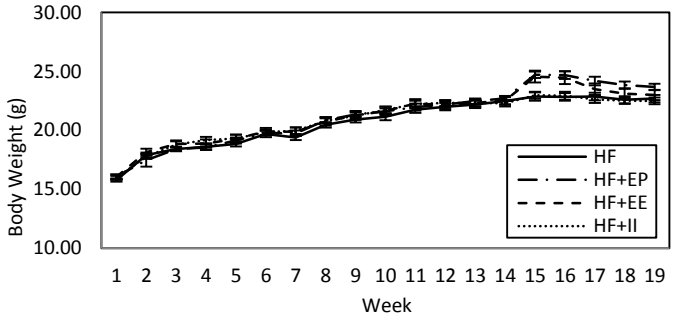


Figure 4.7

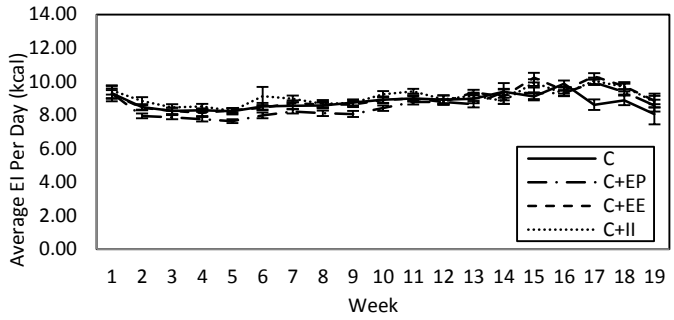


Figure 4.8

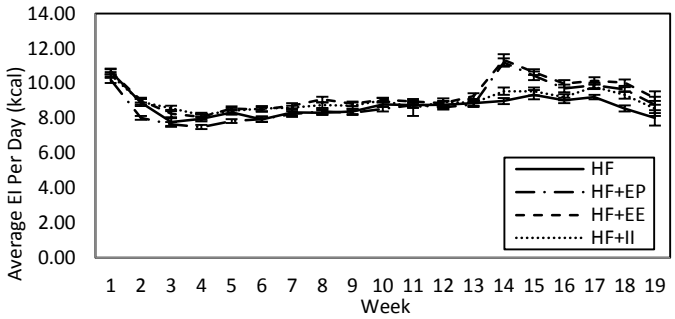


Figure 4.9. Gonadal Adipose Tissue Measurements. Changes in gonadal adipose tissue weight (g) after 4 weeks of feeding epimedium powder (EP), epimedium extract (EE), and icariin (II) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. *p<0.05, N=18.

Figure 4.9

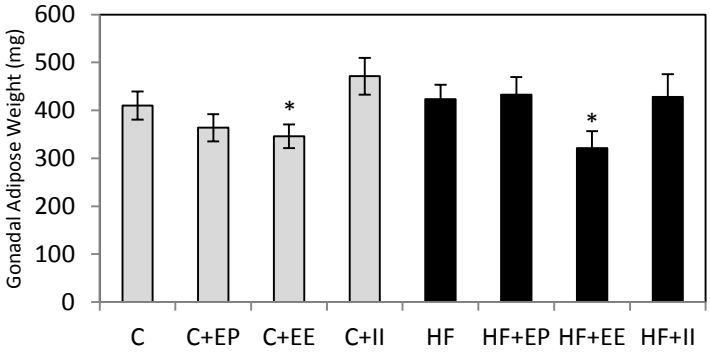


Figure 4.10. Kidney Weight Measurements. Changes in kidney weight (mg) after 4 weeks of feeding epimedium powder (EP), epimedium extract (EE), and icariin (II) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. **p<0.01, N=18.

Figure 4.10

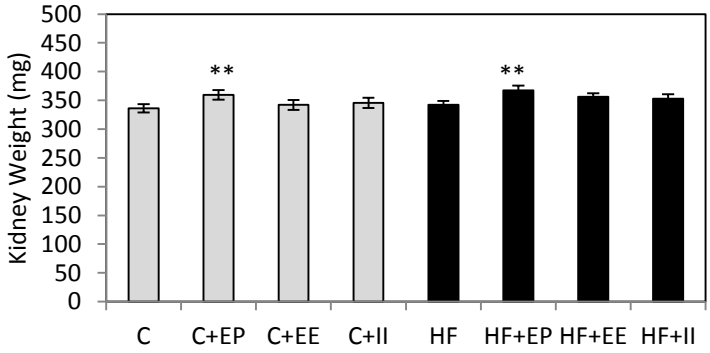
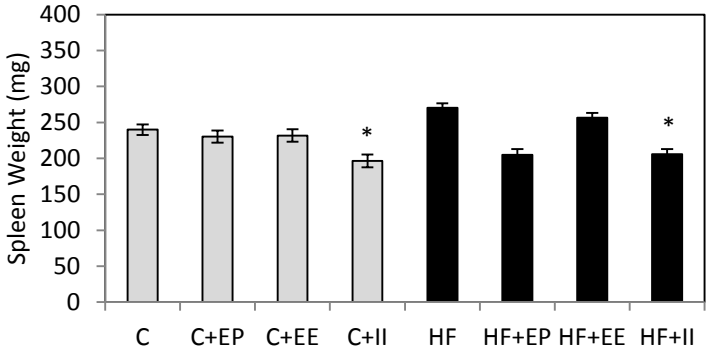


Figure 4.11. Spleen Weight Measurements. Changes in spleen weight (mg) after 4 weeks of feeding epimedium powder (EP), epimedium extract (EE), and icariin (II) with a Control (C) or High Fat (HF) diet. Results are expressed as means +/- SEM. *p<0.05, N=18.

Figure 4.11



CHAPTER 5: SUMMARY & FUTURE DIRECTIONS

Licorice root and epimedium supplements are marketed toward women as an alternative to HRT for menopausal symptom relief. Although some studies have reported them to be effective or beneficial for menopausal symptoms, they may pose a risk due to their phytoestrogenic properties. As evidenced by the results from these studies, licorice root and epimedium are both botanical supplements which are perceived to be safe and beneficial but have very different *in vivo* effects. We investigated the effect of licorice root and epimedium supplementation on late-stage breast cancer metastasis and demonstrated that licorice root consumption has the potential to modulate the degree of metastasis from bone to lung in an animal model while having no effect on the uterus or mammary gland. These effects were only seen with a control diet consisting of 18% kcal from fat and not with a high-fat diet consisting of 45% kcal from fat. In contrast, epimedium compounds appeared to have no effect on metastasis with a control diet. A decrease in metastasis was observed in animals supplemented with epimedium powder in a high fat diet, although this interpretation may be disputable due to the high degree of variability within groups. Epimedium powder and epimedium extract stimulated structures of the uterus and mammary gland, indicating a strong estrogenic effect.

The consumption of botanical phytoestrogenic supplements are viewed to provide the same type of benefits and provide the same type of relief for menopausal symptoms. However, these studies demonstrated that depending on the type of botanical, the phytoestrogenic effects can vary widely and are tissue-specific. Though licorice root and epimedium showed phytoestrogenic effects *in* vivo, the effects were seen in different tissues. The diversity of compounds found in botanical compounds and extracts likely contribute to the differential phytoestrogenic activities. Continued research of other botanicals, as well as licorice root and

epimedium, would help characterize their activities and aid in outlining appropriate usage of various botanicals.

Our current animal is advantageous because it follows a similar pattern of metastasis in humans while maintaining an intact immune system. However, the increased metabolism and obese-resistant phenotype of this model is not as favorable when conducting studies of energy-balance. As seen in our studies as well as others, the responses to a high fat diet in Balb/c mice are dissimilar compared with the responses that would be seen in humans. High fat diets are often high in other macronutrients as well, whereas our high fat diet was formulated to match the control diet as closely as possible in all parameters except fat. In addition, the types of fat incorporated into the AIN-93G diet are not representative of a human diet. Combining the metastatic model used in this study along with the administration of a Western diet may better reflect human outcomes.

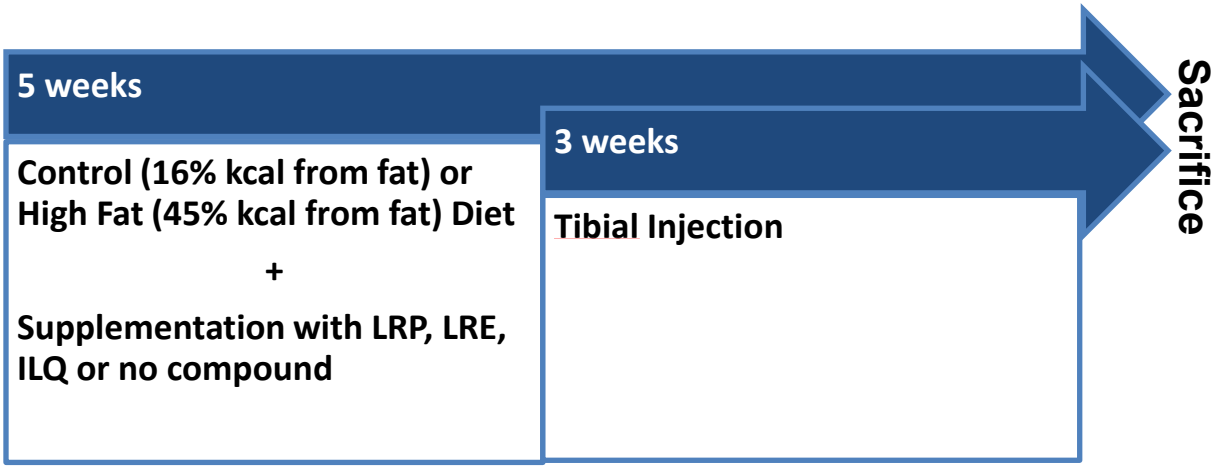
Though our studies were designed to deliver similar amounts of isoliquiritigenin and epimedium, these were broad estimates. Providing animals with diets *ad libitum* added another element of uncertainty. Knowing that these compounds have differing effects at low and high concentrations, determining effects at different doses is vital. Supplying botanicals of a single form in precise amounts of increasing increments ranging from 25-5000 mg/kg BW would help ascertain any dose-dependent relationships.

Of interest are the positive effects of epimedium on bone. We have already established that epimedium has a stimulatory effect on uterus and mammary gland. Many studies have also shown the beneficial effects of epimedium on osteoporosis, which may have implications for growth and metastasis of tumors in the bone. Bone metastasis causes severe pain and increases risk for fractures. Investigation of epimedium supplementation on bone morphology and fracture

risk associated with bone tumor growth would provide insight into the safety of epimedium use on tumors in the bone.

Appendix A

Study Design of Licorice Root Study.



Diet Composition. Composition of Control (C) and High Fat (HF) Diets

	Control AIN-93G (Low-fat)		Modified AIN-93G (High Fat)	
	Gram %	Kcal %	Gram %	Kcal %
Casein	200		240	
L-Cystine	3		3.6	
Corn Starch	397.486		124.683	
Maltodextrin	132		158	
Sucrose	100		119	
Lard	-		155	
Soybean Oil	70		83	
Cellulose	50		59.7	
Mineral Mix	35		42	
Vitamin Mix	10		12	
Choline Bitartrate	2.5		3	
TBHG	0.014		0.017	
Total	1000		974.4	
Protein	17.7	18.8	21.2	18.4
Carbohydrate	60.1	63.9	40.2	34.8
Fat	7.2	17.2	24.0	46.8

Toxicological Evaluation. Summary of Evaluation of Liver, Gallbladder, Kidneys, Spleen and Heart from Histological Slides Stained with H&E in animals fed LRP, LRE and ILQ in a C or HF diet.

ORGANS	HISTOPATHOLOGY	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	average
LIVER	Extra Medullary Hematopoiesis	1	1	1	0	0	0	0	1	1	2	0.7
	Hepatocellular Hypertrophy	0	0	0	0	0	0	0	0	1	0	0.1
	Microgranuloma	1	0	1	0	1	0	1	0	0	0	0.4
	Decreased glycogen content	2	2	0	0	0	2	2	0	2	0	1
GALLBLADDER		/	/	/	/	/	m	/	/	/	/	
KIDNEYS	Capsule mineralization	0	1	0	0	0	0	0	0	0	0	
SPLEEN	Extra Medullary Hematopoiesis	3	4	3	1	1	2	3	3	3	3	2.6
HEART	Epicardial mineralization	2	0	0	0	0	0	0	0	0	0	
TUMOR	injection site (n, y, Y)	Y	y	Y	n	y	y	Y	Y	Y	Y	
	lung metastases (n, y, Y)	y	Y	y	y	y	y	y	Y	Y	y	

ORGANS	HISTOPATHOLOGY	C-LRP-1	C-LRP-2	C-LRP-3	C-LRP-4	C-LRP-5	C-LRP-6	C-LRP-7	C-LRP-8	C-LRP-9	C-LRP-10	average
LIVER	Extra Medullary Hematopoiesis	1	1	2	0	2	1	0	m	3	2	1.3
	Hepatocellular Hypertrophy	1	3	3	3	1	2	1	m	1	1	1.8
	Microgranuloma	1	0	1	0	1	1	1	m	0	0	0.6
	Decreased glycogen content	0	2	0	3	0	2	3	m	2	3	1.7
GALLBLADDER		/	/	/	/	/	/	/	m	/	/	
KIDNEYS		/	/	/	/	/	/	/	m	/	/	
SPLEEN	Extra Medullary Hematopoiesis	3	3	4	3	3	3	3	m	4	m	3.3
HEART		/	/	/	/	/	/	/	m	/	/	
TUMOR	injection site (n, y, Y)	Y	y	y	y	Y	Y	Y	Y	Y	Y	
	lung metastases (n, y, Y)	n	y	y	y	y	y	y	n	y	y	

ORGANS	HISTOPATHOLOGY	C-LRE-1	C-LRE-2	C-LRE-3	C-LRE-4	C-LRE-5	C-LRE-6	C-LRE-7	C-LRE-8	C-LRE-9	C-LRE-10	average
LIVER	Extra Medullary Hematopoiesis	2	1	2	2	1	1	0	0	2	0	1.1
	Hepatocellular Hypertrophy	1	2	0	2	0	1	0	0	0	0	0.6
	Microgranuloma	1	0	0	0	1	0	1	1	0	0	0.4
	Decreased glycogen content	2	0	3	2	2	2	3	3	3	0	2
GALLBLADDER		/	/	/	/	/	/	/	/	/	/	
KIDNEYS	focal infarct	0	0	0	0	1	0	0	2	0	0	
	papillary mineralization	0	0	0	0	1	0	0	0	0	0	
SPLEEN	Extra Medullary Hematopoiesis	3	3	3	2	3	3	3	3	3	1	2.7
HEART		/	/	/	/	/	/	/	/	/	/	
TUMOR	injection site (n, y, Y)	Y	Y	Y	Y	y	Y	Y	Y	Y	n	
	lung metastases (n, y, Y)	y	Y	y	n	y	Y	y	y	y	n	

ORGANS	HISTOPATHOLOGY	C-ILQ-1	C-ILQ-2	C-ILQ-3	C-ILQ-4	C-ILQ-5	C-ILQ-6	C-ILQ-7	C-ILQ-8	C-ILQ-9	C-ILQ-10	average
LIVER	Extra Medullary Hematopoiesis	1	2	2	0	0	0	0	0	1	1	0.7
	Hepatocellular Hypertrophy	1	0	0	0	1	1	0	0	0	0	0.3
	Microgranuloma	1	0	0	0	1	0	1	1	1	1	0.6
	Decreased glycogen content	3	4	2	3	3	4	4	4	4	4	3.5
GALLBLADDER		/	/	/	/	/	/	/	/	/	/	
KIDNEYS		/	/	/	/	/	/	/	/	/	/	
SPLEEN	Extra Medullary Hematopoiesis	2	2	4	3	3	3	3	3	3	4	3
HEART		/	/	/	/	/	/	/	/	/	/	
TUMOR	injection site (n, y, Y)	Y	Y	Y	Y	Y	Y	y	Y	Y	Y	
	lung metastases (n, y, Y)	y	n	n	y	y	y	y	y	n	Y	

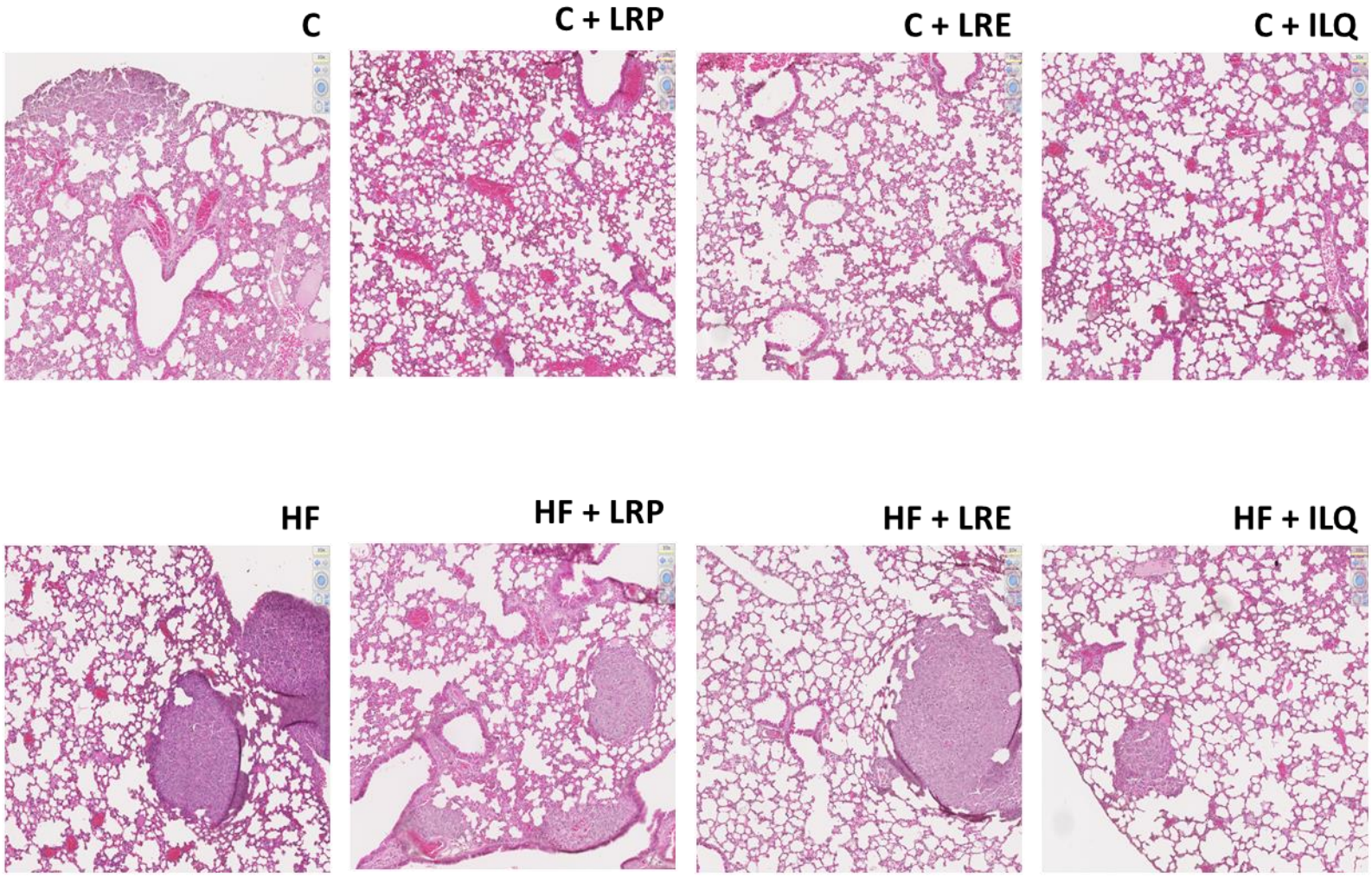
ORGANS	HISTOPATHOLOGY	HF-1	HF-2	HF-3	HF-4	HF-5	HF-6	HF-7	HF-8	HF-9	HF-10	average
LIVER	Extra Medullary Hematopoiesis	0	1	1	1	0	0	0	m	1	1	0.6
	Hepatocellular hypertrophy	0	0	0	0	0	0	0	m	0	0	0.0
	Microgranuloma	0	1	0	0	1	1	0	m	0	1	0.4
	Decreased glycogen content	3	4	3	3	3	3	4	m	3	3	3.2
	Hepatocyte macrovacuolation	0	0	2	0	0	1	0	m	0	0	0.3
GALLBLADDER		/	/	/	/	/	/	/	m	/	/	
KIDNEYS	Chronic progressive nephropathy	0	0	0	0	0	1	0	m	0	0	
SPLEEN	Extra Medullary Hematopoiesis	3	2	3	3	3	2	3	m	3	3	
HEART	Left ventricule cavity: metastasis	0	0	0	0	0	0	0	m	X	0	
	Foci of myocardial fibrosis	0	0	0	0	0	0	0	m	2	0	
	Base of the heart: aortitis	0	3	0	0	0	0	0	m	0	0	
TUMOR	injection site (n, y, Y)	Y	Y	Y	Y	Y	Y	Y	y	Y	Y	
	lung metastases (n, y, Y)	Y	Y	Y	y	y	y	n	y	y	y	

ORGANS	HISTOPATHOLOGY	HF-LRP-1	HF-LRP-2	HF-LRP-3	HF-LRP-4	HF-LRP-5	HF-LRP-6	HF-LRP-7	HF-LRP-8	HF-LRP-9	HF-LRP-10	average
LIVER	Extra Medullary Hematopoiesis	1	1	0	1	1	1	2	0	1	0	0.8
	Hepatocellular Hypertrophy	2	1	3	2	2	3	3	2	3	0	2.1
	Microgranuloma	1	0	1	1	0	0	1	1	0	0	0.5
	Decreased glycogen content	0	1	0	0	1	0	0	0	0	0	0.2
	Hepatocyte macrovacuolation	0	0	0	0	0	0	0	0	0	0	0
GALLBLADDER		/	/	/	/	/	/	/	/	/	/	
KIDNEYS	Chronic progressive nephropathy	0	0	1	0	0	0	0	0	1	0	
SPLEEN	Extra Medullary Hematopoiesis	3	3	3	3	3	3	3	3	2	2	
HEART	Left ventricule cavity: metastasis	0	X	0	0	0	0	0	0	0	0	
TUMOR	injection site (n, y, Y)	Y	Y	Y	Y	y	Y	Y	y	Y	Y	
	lung metastases (n, y, Y)	Y	y	y	Y	y	y	y	Y	y	y	

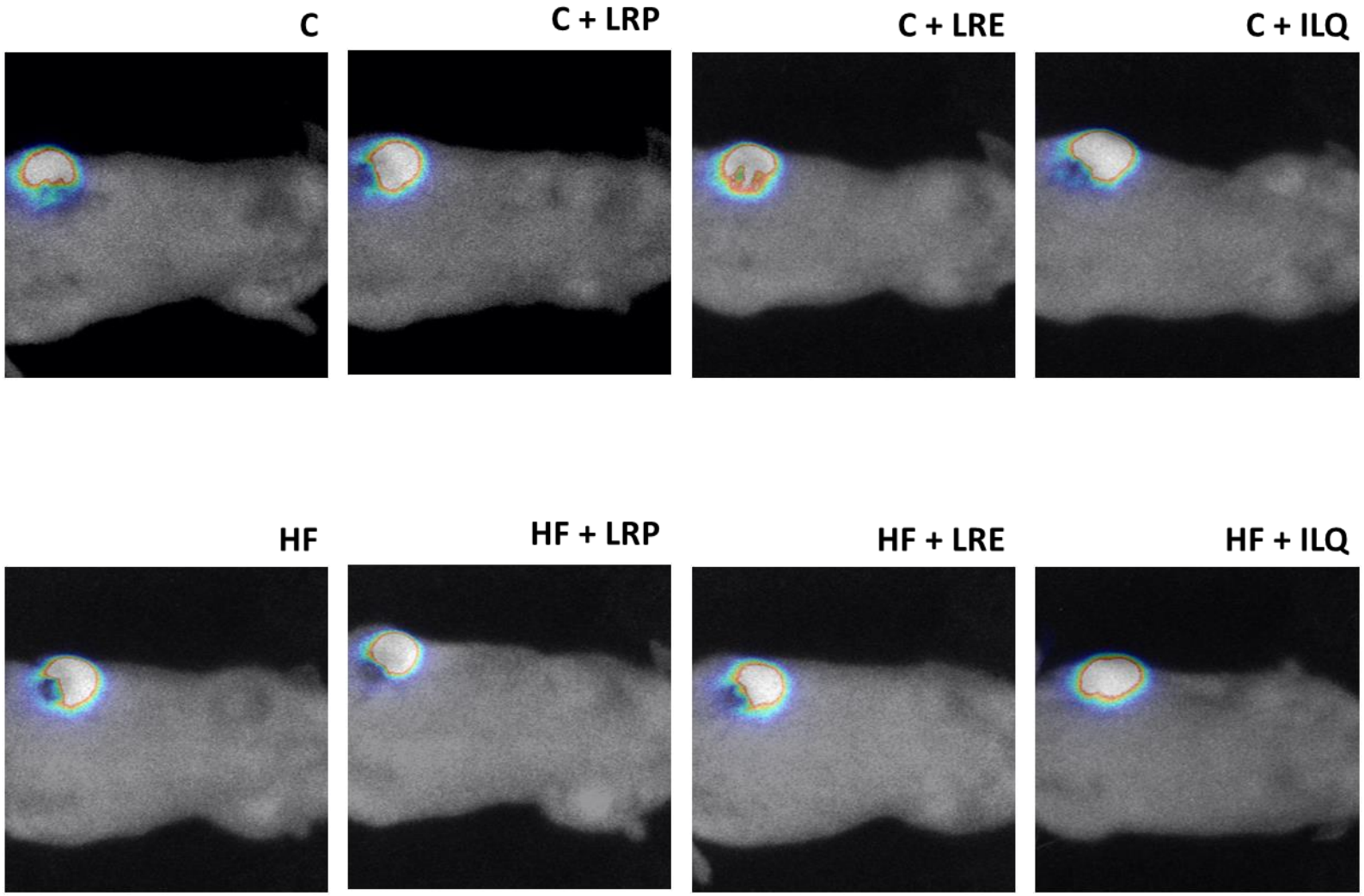
ORGANS	HISTOPATHOLOGY	HF-LRE-1	HF-LRE-2	HF-LRE-3	HF-LRE-4	HF-LRE-5	HF-LRE-6	HF-LRE-7	HF-LRE-8	HF-LRE-9	HF-LRE-10	average
LIVER	Extra Medullary Hematopoiesis	1	1	0	0	0	1	3	1	1	0	0.8
	Hepatocellular Hypertrophy	1	0	2	2	2	0	1	2	0	0	1
	Microgranuloma	1	0	0	1	0	1	1	0	0	0	0.4
	Decreased glycogen content	0	0	0	0	1	1	0	0	1	1	0.4
	Hepatocyte macrovacuolation	0	1	2	1	0	0	0	1	0	0	0.5
	Foci of mineralization	0	0	0	0	1	0	0	0	0	0	0.1
GALLBLADDER		/	/	/	/	/	/	/	/	/	/	
KIDNEYS	Chronic progressive nephropathy	1	0	0	0	0	0	0	0	1	0	
SPLEEN	Extra Medullary Hematopoiesis	3	3	2	3	3	3	3	2	3	3	
HEART	Epicardial mineralization	1	0	0	0	0	0	0	0	0	0	
	Left ventricule cavity: metastasis	0	0	0	0	0	X	0	0	0	0	
TUMOR	injection site (n, y, Y)	Y	Y	Y	Y	Y	Y	y	Y	Y	Y	
	lung metastases (n, y, Y)	Y	Y	y	y	n	y	y	y	y	y	

ORGANS	HISTOPATHOLOGY	HF-ILQ-1	HF-ILQ-2	HF-ILQ-3	HF-ILQ-4	HF-ILQ-5	HF-ILQ-6	HF-ILQ-7	HF-ILQ-8	HF-ILQ-9	HF-ILQ-10	average
LIVER	Extra Medullary Hematopoiesis	0	2	0	1	1	1	0	3	2	1	1.1
	Hepatocellular Hypertrophy	0	1	0	0	2	0	0	2	0	0	0.5
	Microgranuloma	1	1	0	0	0	0	0	0	1	0	0.3
	Decreased glycogen content	0	0	0	0	0	1	0	1	1	1	0.4
	Hepatocyte macrovacuolation	0	0	1	0	0	0	0	2	1	0	0.4
GALLBLADDER		/	/	/	/	/	/	/	/	/	/	
KIDNEYS	Chronic progressive nephropathy	0	0	0	0	0	1	0	0	0	1	
SPLEEN	Extra Medullary Hematopoiesis	3	3	2	3	3	3	3	3	3	3	
	Lymphoid hyperplasia	0	0	0	0	0	0	0	0	0	2	
HEART	Base of the heart: aortitis	0	2	0	0	0	0	0	0	0	0	
TUMOR	injection site (n, y, Y)	Y	Y	y	Y	y	Y	y	y	y	Y	
	lung metastases (n, y, Y)	Y	y	y	Y	Y	Y	Y	y	y	Y	

Lung Tumors Histology. Representative Images of Histological Lung Sections Stained with H&E at 10x magnification in lungs of animals supplemented with LRP, LRE and ILQ in a C or HF diet.

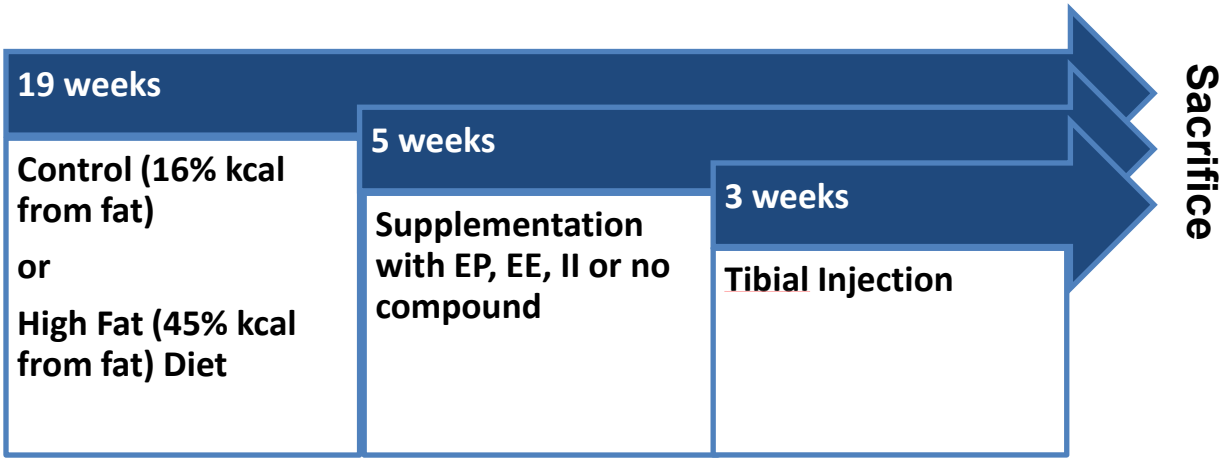


Bioluminescence Imaging (BLI). Representative Images of Tibial Tumors imaged with BLI on Day 8 after Injection in animals supplemented with LRP, LRE and ILQ in a C or HF diet.



Appendix B

Study Design of Epimedium Study.



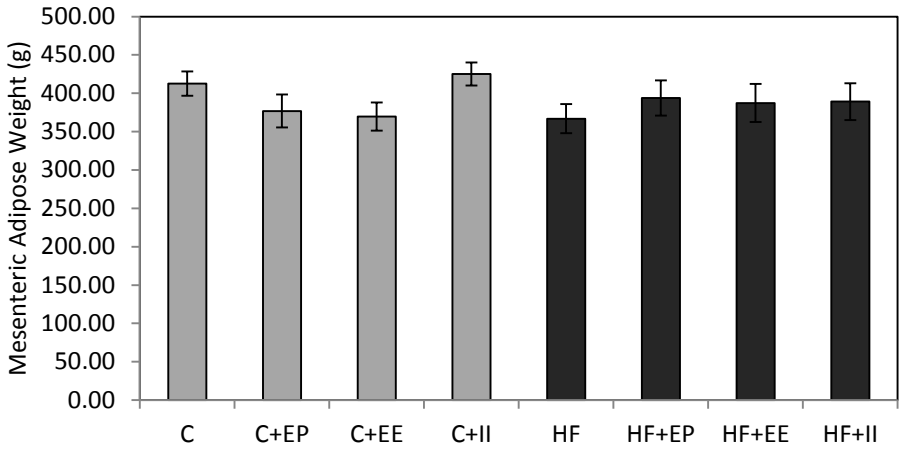
Diet Composition. Composition of Control (C) and High Fat (HF) Diets

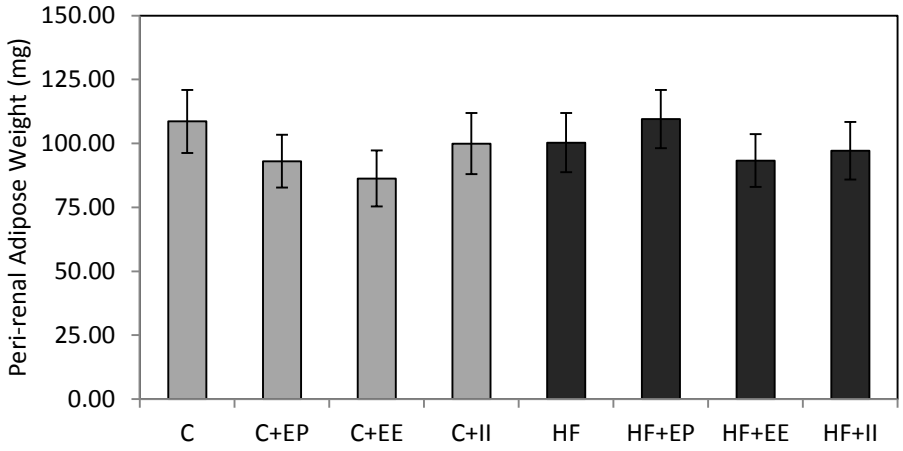
	Control AIN-93G (Low-fat)		Modified AIN-93G (High Fat)	
	Gram %	Kcal %	Gram %	Kcal %
Casein	200		240	
L-Cystine	3		3.6	
Corn Starch	397.486		124.683	
Maltodextrin	132		158	
Sucrose	100		119	
Lard	-		155	
Soybean Oil	70		83	
Cellulose	50		59.7	
Mineral Mix	35		42	
Vitamin Mix	10		12	
Choline Bitartrate	2.5		3	
TBHG	0.014		0.017	
Total	1000		974.4	
Protein	17.7	18.8	21.2	18.4
Carbohydrate	60.1	63.9	40.2	34.8
Fat	7.2	17.2	24.0	46.8

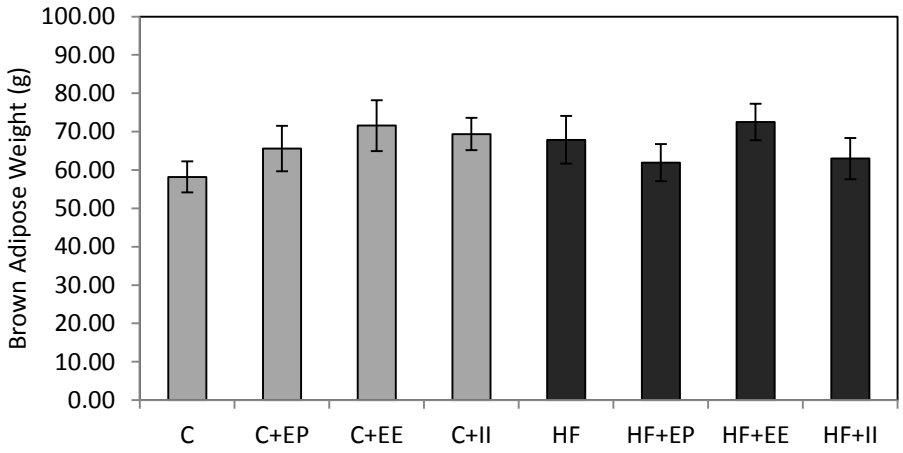
Levels of Icariin and Derivatives in Feed. Levels of Icariin (II), icariside I (ICARI), icariside II (ICARII), desmethylicaritin (DICT), and icaritin (ICT) detected in pelleted feed.

		FEED				
PERCENTAGE IN FEED	SAMPLE	TOTAL II (MG/G)	TOTAL ICARI (MG/G)	TOTAL ICARII (MG/G)	TOTAL DICT (MG/G)	TOTAL ICT (MG/G)
-	**CONTROL**	LOD	LOD	LOD	LOD	LOD
5%	**C-EP**	0.27	0.007	0.034	LOD	0.009
0.50%	**C-EE**	0.22	0.006	0.030	LOD	0.007
0.05%	**C-II**	0.41	0.005	0.001	LOD	LOD
-	**HIGH FAT**	LOD	LOD	LOD	LOD	LOD
5%	**HF-EP**	0.20	0.005	0.027	LOD	0.006
0.50%	**HF-EE**	0.20	0.005	0.025	LOD	0.006
0.05%	**HF-II**	0.04	0.0005	LOD	LOD	LOD

Adipose Tissue Weights. Mesenteric Adipose Tissue (MAT), Peri-renal Adipose Tissue (PAT), and Brown Adipose Tissue (BAT) Weights after Supplementation with EP, EE, and II in a C or HF diet.







Bioluminescence Imaging (BLI). Representative Images of Tibial Tumors imaged with BLI on Day 8 after Injection in animals supplemented with EP, EE and II in a C or HF diet.